MEJORA DE LA FUNCIONALIDAD DE PROTEÍNAS DE CEREALES LIBRES DE GLUTEN: APLICACIÓN EN PRODUCTOS FERMENTADOS

Tesis Doctoral

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RESUMEN

La enfermedad celíaca (EC) es una intolerancia crónica a las proteínas del gluten cuyo único tratamiento es el seguimiento de una dieta libre de gluten durante toda la vida, ya que su ingesta provoca una lesión en la mucosa del intestino delgado, disminuyendo la absorción de los nutrientes (proteínas, grasas, vitamina B12, ácido fólico, hierro, calcio y otros nutrientes), produciendo carencias nutricionales. El principal inconveniente de los productos fermentados libres de gluten es la ausencia de proteínas con las propiedades viscoelásticas del gluten. Otro inconveniente es su bajo contenido proteico, ya que la mayoría de los productos que se encuentran actualmente en el mercado están compuestos en su mayor parte por almidón. Por ello, se propone el enriquecimiento proteico de los productos fermentados libres de gluten y el uso de coadyuvantes tecnológicos que permitan modificar la funcionalidad de sus proteínas así como la mejora de la calidad del producto. En este trabajo se estudió el efecto de la adición de aislados proteicos de diversas fuentes (soja, guisante, albumen de huevo y suero lácteo), sobre las propiedades reológicas y funcionales de las masas de arroz, y la posible creación simultánea de una red proteica catalizada por la enzima transglutaminasa (TG). Las proteínas de soja y guisante son las que originaron masas con adecuadas propiedades viscoelásticas para el proceso de panificación. Sin embargo, la combinación de ambas no provocó efectos sinérgicos sobre las propiedades reológicas y funcionales de las masas de harina de arroz adicionadas con estos aislados proteicos. Los resultados obtenidos se extrapolaron al proceso de panificación, seleccionándose la soja para el diseño de productos libres de gluten enriquecidos en proteína. El uso de hidroxipropilmetilcelulosa (HPMC) permitió la mejora de la estructura de los productos fermentados libres de gluten, obteniendo un mejor volumen y una miga más aireada y más próxima a la que se obtiene con trigo. La adición de transglutaminasa también mejoró las características tecnológicas de los panes sin gluten enriquecidos en proteína. La adecuación de la formulación y el proceso de panificación ha permitido obtener un pan libre de gluten enriquecido en proteína de soja y con unas características tecnológicas adecuadas.

ABSTRACT

Celiac disease is a chronic intolerance to the gluten proteins and the only treatment is to keep a gluten-free diet for all the life, since its ingestion damages the small intestine mucous, decreasing the nutrient absorption (proteins, lipids, B12 vitamin, folic acid, iron, calcium and other nutrients), producing nutritive deficiencies. The main inconvenient of the fermented gluten-free products is the absence of proteins with viscoelastic properties like gluten. Another inconvenient is the low protein content, since the starch is the major component in most of the commercial products. Therefore, it is proposed the protein enrichment of the gluten-free fermented products and the use of processing aids, in order to modify the functional properties of the proteins and to improve the product quality. The effect of the addition of protein isolates from different sources (soybean, pea, egg albumen and whey) on the rheological and functional properties of the rice doughs and the possible formation of a protein network by the use of transglutaminase (TG) was studied. Soybean and pea protein produced doughs with better viscoelastic properties for the breadmaking process. However, the addition of both proteins simultaneously did not produce synergistic effects on the rheological and functional properties of the rice doughs. The results were extrapolated to a breadmaking process, selecting the soybean protein for designing protein enriched gluten-free products. The use of hydroxypropyl-methylcellulose (HPMC) allowed improving the structure of the fermented gluten-free products, obtaining better volume and a more aerated crumb, closer to the obtained with wheat. The addition of TG also improved the technological properties of the protein enriched gluten-free breads. The optimization of the formulation and the breadmaking process has allowed to obtain a soybean enriched gluten-free bread and with appropriate technological properties.

ÍNDICE

Introducción 1

Unidad Temática: *Rice* 13

Objetivos y plan de trabajo 47

Capítulo 1: *Effect of different protein isolates and transglutaminase on rice flour properties* 51

Capítulo 2: *Effect of transglutaminase on the protein pattern of rice,soybean and their blends* 75

Capítulo 3: *Effect of microbial transglutaminase on the protein fractions of rice, pea and their blends* 99

Capítulo 4: *Functional and rheological properties of protein enriched gluten free composite flours* 119

Capítulo 5: *Breadmaking performance of protein enriched gluten free breads* 147

Discusión general 173

Conclusiones 181

Introducción

Ya en la segunda mitad del siglo II a. de C., Arateus de Capadocia hizo las primeras descripciones de la enfermedad celíaca de las que se tiene constancia. En sus textos griegos describió la diarrea grasa, pérdida de peso, palidez, diarrea crónica y recidivante, observados en pacientes cuyo estómago no retenía los alimentos y pasaban a través de él sin ser digeridos, y sin que el organismo pudiera asimilar nada. E incluso apunta que el pan es raramente adecuado para proporcionar energía a los niños que padecían esta enfermedad. El nombre que se daba a los pacientes de esta enfermedad era el de *"koliacos"* (celíacos), cuyo significado era *"aquéllos que sufren del intestino"*. Pero no es hasta 1950 cuando el holandés Willem Karen Dike descubre que el gluten es el causante de esta enfermedad.

La enfermedad celíaca (EC) es una intolerancia crónica a las proteínas del gluten y cuyo único tratamiento es el seguimiento de una dieta libre de gluten de por vida. La ingesta de gluten desencadena una reacción inmunológica en el intestino de personas predispuestas a padecer la enfermedad. Esto provoca una lesión en la mucosa del intestino delgado proximal, disminuyendo la superficie disponible para la absorción de nutrientes, produciendo una malabsorción de los mismos y, por tanto, carencias nutricionales (proteínas, grasas, vitamina B12, ácido fólico, hierro, calcio y otros nutrientes). Las manifestaciones clínicas de esta enfermedad son muy variadas. La utilización de técnicas de screening, como los marcadores serológicos, para la detección de nuevos casos de celiaquía, ha permitido advertir que esta enfermedad es mucho más frecuente de lo que se creía, situándose la incidencia en Europa en torno a 1/100-300 (Holmes, 2001; Corrao y col., 2001; Wahab y col., 2002). La manifestación clásica es una enteropatía o lesión intestinal y se caracteriza por la aparición de diarrea, malnutrición, distensión abdominal, rechazo del alimento

y carácter huraño. Sin embargo, otras veces la EC se manifiesta de forma menos clara, sin síntomas digestivos. En este caso se habla de formas atípicas de la enfermedad, cuyos síntomas pueden ser retraso de talla, anemia ferropénica refractaria, esterilidad, alteraciones de piel, vitíligo, osteoporosis, leucoencefalopatía, epilepsia, hipoplasia del esmalte dental, calcificaciones cerebrales, daño hepático, hipoproteinemia e hipoprotrombinemia. Por otra parte, existen enfermedades asociadas a la EC, como diabetes mellitus, dermatitis herpetiforme, síndrome de Down y enfermedades neurológicas. La amplia variedad de manifestaciones de esta enfermedad llevó a proponer el modelo iceberg para la representación de la prevalencia de la EC, donde la prevalencia viene representada por el iceberg completo (Fig. 1). Los enfermos con síntomas clásicos de enfermedad celíaca son solamente la punta del iceberg de todos los sujetos que realmente están afectados, aunque no hayan sido diagnosticados. Por debajo de la línea del agua se situarían los sujetos con enfermedad celíaca silente, donde se incluyen los casos de enteropatía sensible al gluten que no dan sintomatología aunque la biopsia intestinal está alterada y la genética sea positiva, como es el caso de familiares de niños con enfermedad celíaca. Estos sujetos no han sido diagnosticados porque no muestran síntomas o los síntomas que muestran no se han asociado con la enfermedad celíaca. Debajo de éstos encontramos un grupo de pacientes con enfermedad celíaca latente. En este grupo se encuentran aquéllos individuos genéticamente predispuestos que tienen una biopsia normal aunque tomen gluten pero, o bien previamente fueron diagnosticados ya de enfermedad celíaca con una biopsia, cuya lesión se recuperó o bien se les hizo previamente un estudio en el que se demostró que no eran celíacos (por ejemplo familiares de un enfermo), pero en una época posterior aparece la lesión típica de la enfermedad celíaca. Esto indica que una biopsia intestinal normal en un sujeto que está consumiendo gluten no descarta

definitivamente la enfermedad. Y por último, en la base del iceberg se encontrarían aquéllos sujetos con enfermedad celíaca potencial.

En este grupo se incluyen aquellos individuos que tienen una predisposición genética a padecer la enfermedad, pero que no tienen ningún dato real para el diagnóstico.

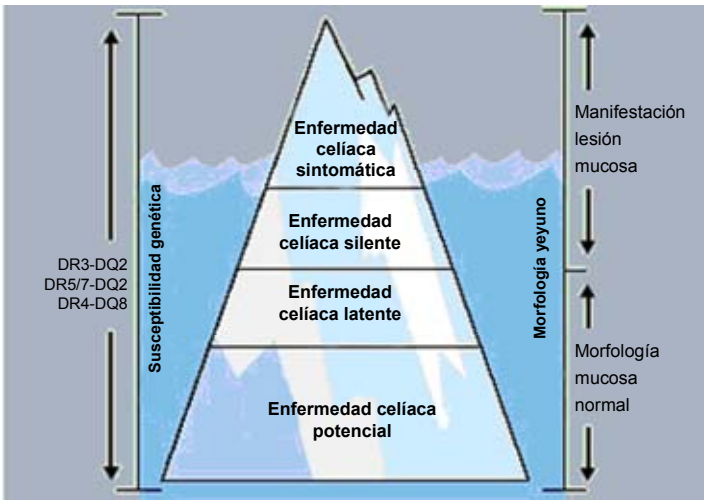

Fig. 1. Iceberg de la enfermedad celíaca (Mäki y Collin, 1997).

En la enfermedad celíaca se ven involucrados tres factores: el gluten, la susceptibilidad genética HLA (antígenos leucocitarios humanos) y factores ambientales. El gluten es el que desencadena la reacción inmunológica en sujetos genéticamente predispuestos. En cuanto a la susceptibilidad genética, todos los enfermos celíacos tienen un determinado tipo de HLA, aunque no todas las personas que lo tienen presentan la enfermedad, por lo que otros genes podrían estar también implicados en la aparición de la enfermedad. Y, respecto al último factor, parece que

determinados procesos ambientales, como la introducción precoz de gluten, podrían influir en el inicio de aparición de la enfermedad.

Como se ha comentado anteriormente, actualmente el único tratamiento es la eliminación del gluten de la dieta durante toda la vida, ya que cuando éste se deja de consumir, mejora la sintomatología y paulatinamente se recupera la mucosa intestinal (Corrao y col., 2001). Sin embargo, la introducción del gluten en la dieta revierte el cuadro clínico y aparecen de nuevo las lesiones intestinales. Los péptidos liberados en la digestión del gluten de trigo son los responsables primarios de esta intolerancia en personas genéticamente predispuestas. Aunque se desconoce el mecanismo exacto de esta enteropatía, los cereales que contienen gliadinas o prolaminas se consideran tóxicos para los celíacos (trigo, centeno, cebada y probablemente avena), siendo las gliadinas más tóxicas aquéllas que presentan un alto contenido en los aminoácidos prolina y glutamina. El arroz, maíz, sorgo, mijo y trigo sarraceno son considerados cereales aptos para los celíacos.

Tradicionalmente, los productos dirigidos a la población celiaca se diseñaban atendiendo únicamente a la ausencia de alergenos, utilizando mezclas de polímeros que pudieran originar productos con características sensoriales similares a los que contienen gluten. En los últimos años, el colectivo celiaco ha atraído la atención de las empresas de alimentación y tecnólogos de alimentos, y se han desarrollado una gran variedad de productos sin gluten. En el caso de productos de panadería, la diversidad de productos comerciales obedece principalmente a la introducción de numerosos formatos y presentaciones más que al diseño de nuevos productos con características distintas. Los productos de panadería sin gluten disponibles en el mercado se caracterizan por estar constituidos por mezclas de almidones (fundamentalmente de maíz) y harina de cereales sin gluten (arroz o maíz); además, en ocasiones se utilizan fibras o proteínas de diversos orígenes (huevo, soja), lo que

origina productos ricos en hidratos de carbono pero deficientes en proteínas (Tabla 1). De hecho, la composición de los productos de panadería sin gluten que se encuentran en el mercado varía entre 35-45% de hidratos de carbono, 2.5-6.0% de proteínas, 2.0-10.0% de lípidos y cantidades minoritarias de fibras y minerales; perfil que difiere de los productos de panadería con gluten, cuya composición varía entre 41-56% de hidratos de carbono, 8.0-13.0% de proteínas y 2.0-4.0% de grasas, entre sus constituyentes mayoritarios (Collar, 2007). El diseño y desarrollo de productos de panadería sin gluten con un mayor contenido proteico permitiría disponer de productos alternativos y nutritivamente enriquecidos, adecuados para paliar deficiencias que pudieran derivarse de la enfermedad celiaca.

El arroz se encuentra entre los cereales más adecuados para el desarrollo de productos sin gluten, ya que su harina se caracteriza por poseer un sabor suave, color blanco, es fácilmente digerible y tiene propiedades hipoalergénicas. Además posee bajos niveles de sodio e hidratos de carbono fácilmente digeribles (información más detallada sobre las propiedades y aplicaciones del arroz se incluyen en la unidad temática *Rice*). A pesar de las características beneficiosas de la harina de arroz, ésta se utiliza como ingrediente minoritario en los productos de panadería comerciales. Dado que la harina de arroz es un subproducto de las industrias arroceras, el desarrollo de productos de panificación sin gluten basados en harina de arroz sería una alternativa para dar valor añadido a este subproducto.

Desde el punto de vista tecnológico el desarrollo de productos de panadería sin gluten requiere la incorporación de ingredientes poliméricos que mimeticen la funcionalidad de la red de gluten, matriz viscoelástica capaz de retener el gas que se desprende durante la fermentación en el proceso de panificación, y principal

Tabla 1. Composición de pan sin gluten existente en el mercado.

Marca comercial	Tipo de pan	Harina	Almidón	Otros ingredientes	Espesante	Emulgente	Otros aditivos	Agentes leudantes
PROCELI	Baguette		Maíz	Agua, azúcar, sal	Goma xantana	E-472e	Propionato cálcico, ácido L-ascórbico	Levadura, gasificantes (difosfato disódico, bicarbonato sódico)
	Baguette mediterránea		Maíz	Agua, azúcar, sésamo tostado, aceite de oliva virgen (1,95%), sal	Goma xantana	E-472e	Propionato cálcico, ácido L-ascórbico	Levadura, gasificantes (difosfato disódico, bicarbonato sódico)
	Pan		Maíz	Agua, azúcar, sal	Goma xantana	E-472e	Propionato cálcico, ácido L-ascórbico	Levadura, gasificantes (difosfato disódico, bicarbonato sódico)
	Molde arroz	Arroz (17,16%)	Maíz, tapioca	Agua, margarina vegetal, azúcar, sal	Goma xantana	E-472e	Propionato sódico, ácido L-ascórbico	Levadura, gasificantes (difosfato disódico, bicarbonato sódico)
	Molde arroz integral	Arroz integral (14,6%)	Tapioca	Agua, margarina vegetal, azúcar, sal	Goma xantana	E-472e	Propionato sódico, ácido L-ascórbico	Levadura, gasificantes (difosfato disódico, bicarbonato sódico)
	Molde inglés		Maíz	Agua, azúcar, huevo, margarina vegetal, sal	Goma xantana	E-472e	Propionato sódico, ácido L-ascórbico	Levadura, gasificantes (difosfato disódico, bicarbonato sódico)
La Colegiala	Bollo s/gluten s/margarina y s/huevo	Arroz	Maíz	Agua, fibra vegetal, sal.				Levadura fresca
	Pan fresco s/gluten	Arroz	Maíz	Agua, margarina vegetal, huevo pasteurizado, azúcar, fibra vegetal, sal marina				Levadura fresca
	Pan fresco s/gluten y s/huevo	Arroz	Maíz	Agua, margarina vegetal, azúcar, fibra vegetal, sal marina.				Levadura fresca
	Bollo s/gluten	Arroz	Maíz	Agua, margarina vegetal, huevo pasteurizado, fibra vegetal, sal				Levadura fresca
	Bollo s/gluten redondo	Arroz	Maíz	Agua, margarina vegetal, huevo pasteurizado, semillas de sésamo, fibra vegetal, sal				Levadura fresca
Schär	Chapata	Maíz, arroz	Maíz	Agua, fibra vegetal, dextrosa, aislado proteico de soja, aceite vegetal, sal	HPMC		Ácido cítrico, ácido tartárico	Levadura
	Baguette	Arroz	Maíz	Agua, dextrosa, psyllium, aislado de proteínas de soja, aceite vegetal, sal	HPMC			Levadura
	Pan carré	Arroz	Maíz	Agua, aceite vegetal, azúcar, proteínas vegetales, sal, fibras vegetales	Harina de semilla de guar, HPMC	E-472e		Levadura

responsable de la estructura de la miga del pan. Los compuestos poliméricos generalmente usados como sustitutos del gluten incluyen las gomas o hidrocoloides (goma guar, goma xantana, carragenanos, agar, goma de garrofín, hidroxipropilmetilcelulosa) (Gujral y col., 2003; Gujral y Rosell, 2004a, 2004b; Sivaramakrishnan, Senge y Chattopadhyay, 2004; McCarthy y col., 2005), o bien almidones de distinta naturaleza (maíz, patata, tapioca) (Nishita, Roberts y Bean, 1976; Kang, Choi y Choi, 1997; Kobylañski, Pérez y Pilosof, 2004; Schober, Bean y Boyle, 2007; Lazaridou, Duta y Papageorgiou, 2007). Sin embargo, la utilización de proteínas como ingrediente polimérico estructural en productos libres de gluten no ha sido suficientemente explorada. Algunos de los productos libres de gluten comerciales contienen proteínas de huevo, leche o soja en cantidades reducidas, adicionadas por su papel nutritivo en este tipo de productos y en algunos casos ayudan a mejorar el volumen, apariencia y características sensoriales (Gallagher y col., 2003; Ribotta y col., 2004; Moore y col., 2006). Sin embargo, la inclusión de ingredientes proteicos en los productos de panadería sin gluten podría explotarse con una doble finalidad, atendiendo a su valor nutritivo y a su posible funcionalidad estructural en la producción de productos fermentados.

La transglutaminasa (TG) (EC 2.3.2.13) es una enzima que cataliza la reacción entre un grupo ε-amino de un residuo de lisina y un grupo γ-carboxiamida de un residuo de glutamina, obteniendo un entrecruzamiento covalente de las proteínas, que puede ser inter- o intramolecular. Aunque esta es la reacción dominante, la TG también cataliza otras reacciones. En presencia de aminos primarios, la TG cataliza el entrecruzamiento entre el grupo amino y un grupo γ-carboxiamida de un residuo de glutamina. En ausencia de substratos amino, la TG cataliza la hidrólisis del grupo γ-carboxiamida de la glutamina, produciéndose la deamidación. Se ha demostrado la actuación de esta enzima sobre proteínas de naturaleza muy diversa como las del

gluten de trigo, proteínas de soja, proteínas de suero lácteo, miosina y actomiosina (Zhu y col., 1995; Bonet, Blaszczak y Rosell, 2006), obteniéndose estructuras poliméricas con propiedades reológicas y funcionales modificadas, lo cual permite ampliar sus aplicaciones en alimentos. En el caso concreto de productos derivados de cereales, la TG se ha utilizado para mejorar la calidad panadera de harinas de trigo débiles o dañadas (Autio y col., 2005; Bonet y col., 2005; Caballero y col., 2005), consiguiendo mejorar la textura del pan (Collar, Bollaín y Angioloni, 2005; Gerrard y col., 1998).

El diseño y desarrollo de productos de panadería libres de gluten basados en harina de arroz y enriquecidos en proteínas compatibles y complementarias, desde el punto de vista tecnológico y nutricional, a las proteínas de origen cereal, aportaría a la población celíaca un producto alternativo y nutricionalmente mejorado. Los diversos aislados proteicos comerciales, así como la actividad entrecruzante de la enzima transglutaminasa, constituyen herramientas esenciales para explotar la vertiente estructural de las proteínas, necesaria para el desarrollo de productos panarios fermentados.

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Unidad Temática

Rice

Rice

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INTRODUCTION

Rice has been, throughout history, one of the most important foods in the human diet and one of the most extended cereal crops (9% of the total cultivated soil). In fact, rice has probably fed more people in history than any other crop. Even today, rice grains sustain two thirds of the world's population, approximately 2.5 billion people. However, around the world, the contribution that rice makes to diet differs and the types of processing involved are also quite different. Rice is mainly consumed as white grain, but in the last decade dozens of products containing rice as an ingredient have appeared on the food market. Two different species of rice are cultivated: *Oryza sativa* and *Oryza glaberrima*, and there are around 22 wild species. *Oryza sativa* originated in the wet tropic of Asia, but is now cultivated around the world, whereas *Oryza glaberrima* has been cultivated in West Africa for the last 3500 years.

Rice accounts for 29% of the world's total cereal production, and is comparable to the production of wheat and corn. Cultivation is concentrated in the developing countries, mainly around East and Middle Asia, where 91% of the total world production is located (FAOSTAT, 2007) (Figure 1). China is the world's largest rice producer (30%), followed by India (21%), Indonesia (9%) and Bangladesh (6%). The rest of Asia, America and Africa produce 37, 5 and 3%, respectively of the total world rice production. The amount of rice and rice-based products available for human

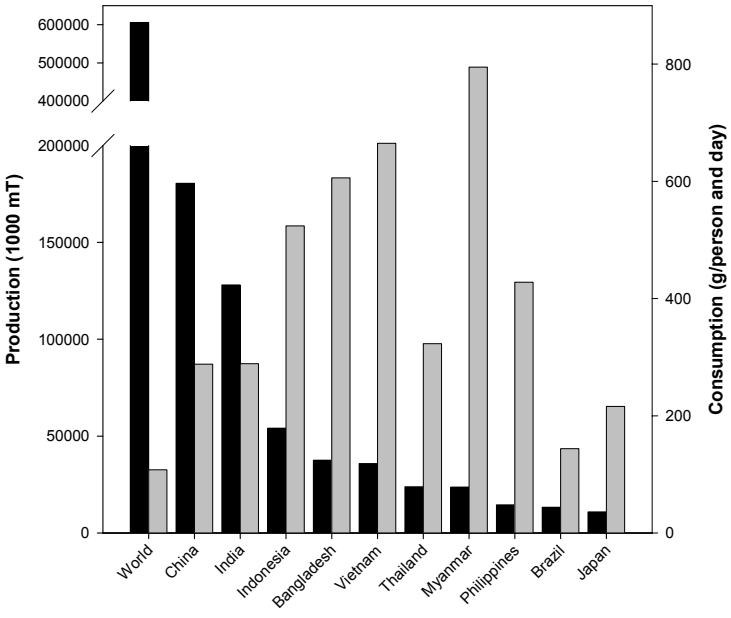

Figure 1. Paddy rice production (black bars) and their consumption (grey bars) among the world's largest rice producers. Values are expressed in grams per person and per day. Data: FAOSTAT, 2004.

consumption in the different countries is almost parallel to the rice production. With minor exceptions, practically all the rice production is consumed within the producers' countries. The highest daily rice consumption is observed in Myanmar, with 795 g per capita. The average daily consumption of rice in the Asian countries is 285 g per capita, ahead of the 44g per capita of rice consumed in the developed countries. Nowadays, there are three big models of rice consumption (Infocomm, 2007): the Asian model, with an average yearly consumption higher than 80 kg per person; the

subtropic developing countries model with a consumption between 30 and 60 kg per person; and the Occident model with a consumption lower than 10 kg per person. In the last decade, rice consumption experienced a steady decrease in the developed countries; this tendency promoted the development of new and innovative rice-based products. Remarkably, in 2000 over 400 new products containing rice were placed on the market (Wilkinson and Champagne, 2004), as a result of new initiatives designed to increase rice consumption.

Rice provides 27% of the total energy intake in the developing countries, and only 4% of the total energy intake in the developed countries. Like other cereals, rice is a cheap source of proteins, and in developing countries, rice supplies 20% of the dietary protein intake.

The composition of the rice grain depends on the cultivars grown, environmental factors, and processing. Rice can be cultivated in diverse conditions, although it grows faster in wet and warm environments. Rice grains can be short, medium or long. They can be sticky or glutinous and non-sticky, and can be a variety of different colours, including black to red and brown; some species are even aromatic. Rice grains without the hull are brown due to the colour of the three-pericarp layers that cover the grain. The rice grain is rich in complex carbohydrates, and represents a source of proteins, minerals and vitamins, mainly B vitamins, and does not contain cholesterol (Table 1). The chemical composition of the rice grain changes during milling. Removal of the outer bran layers causes a loss of proteins, fats, and a large percentage of the fiber, vitamins, and minerals. Iron, phosphorous, potassium and magnesium are the most important minerals in this cereal. The hull represents 20% of the grain and is composed of silica and hemicelluloses (Champagne *et al.,* 2004).

Table 1. Rice composition and energetic value of rice grain and rice flour (referred to 100g).

		Rough rice	Milled rice	Whole meal flour	White flour
Carbohydrates (%)		77.2	79.9	76.5	80.1
Proteins (%)		7.9	7.1	7.2	5.9
Dietetic fibre (%)		3.5	1.3	4.6	2.4
Lipids (%)		2.9	0.7	2.8	1.4
Minerals (%)		1.5	0.6	1.5	0.6
Lipids	Saturated (g)	0.6	0.7	2.8	1.4
	Mono-unsaturated (g)	1.1	0.2	1.0	0.4
	Poly-unsaturated (g)	1.0	0.2	1.0	0.4
Minerals	Calcium (mg)	23.0	28.0	11.0	10.0
	Iron (mg)	1.5	0.8	2.0	0.4
	Magnesium (mg)	143.0	25.0	112.0	35.0
	Phosphorus (mg)	333.0	115.0	337.0	98.0
	Potasium (mg)	223.0	115.0	289.0	76.0
	sodium (mg)	7.0	5.0	8.0	0.0
Vitamins	E vitamin (mg)	1.2	0.1	1.2	0.1
	K vitamin (mg)	1.9	0.1	---	0.0
	Thiamine (mg)	0.4	0.1	0.4	0.1
	Riboflavin (mg)	0.1	0.0	0.1	0.0
	Niacin (mg)	5.1	1.6	6.3	2.6
	Pyridoxine (mg)	0.5	0.2	0.7	0.4
	Folate (µg)	20.0	8.0	16.0	4.0
	Pantothenic acid (mg)	1.5	1.0	1.6	0.8

Source: Rosell *et al.* (2007b)

Carbohydrates are the most abundant component in rice, with starch contents reaching approximately 80% (14% moisture). Rice starch is a glucose polymer composed by amylose and amylopectin in different proportion depending on the rice variety. The content of starch in the rice grain increases from the surface to the core, and thus milled rice is rich in starch. Rice starch is considered not allergenic because of the hypoallergenic proteins present. Starch determines the physical properties and functionality of the rice grains, and these properties are greatly dependent on the amylose/amylopectin ratio. Amylopectin is the branched polymer, more abundant than the linear polymer (amylose). However, amylose has received more attention from the scientific community because it is considered an indicator of cooking quality. Rice starch that lack amylose is called “waxy”, because its mutation at the waxy locus, or “glutinous”, due to its opaque appearance. Complete information about rice starch structure and functional properties has been recently reviewed by Fitzgerald (2004).

Protein is the second most abundant constituent of milled rice, ranging from 6.3-7.1 g of N x 5.95. Protein concentration decreases from the surface to the center of the kernel, and they are deficient in the essential amino acid lysine. The albumin, globulin, prolamin, and glutelin content is unique among the cereals, with a high concentration of glutelins, and a low of prolamins (Hamaker, 1994). This characteristic determines the high content of lysine when compared to other cereals. The most abundant essential amino acids are glutamic acid, aspartic acid, leucine and arginine, followed by alanine, valine, phenylalanine and serine. Lipids are minor components, but they contribute to the nutritional, sensoric and functional characteristics, since they form complexes with the amylose chains. Rice lipids are classified in starchy and non-starchy lipids. The majority of the lipids are non-starchy lipids, and they are located in the aleurone layer and germ. They comprise neutral lipids, with a small amount of glycolipids and phospholipids. Recently, some minor

lipids have been related to the role of rice in the prevention of chronic diseases like cancer and heart diseases (Watkins *et al.,* 1990).

PRODUCTION OF RICE FLOURS AND THEIR PROPERTIES

Rice flour production

Milling

Rice is harvested and threshed to produce the so-called “paddy” or “rough” rice, where the kernel is still within the hull or husk. As for wheat, milling is the usual method used to process rice, although the term “milling” in the rice industry is used for a process that is completely different from wheat milling. Wheat is milled to obtain flour, whereas milling of rice comprises the removal of the husk, stripping the bran of the endosperm, and finally removing broken and altered kernels. Milling of rice drastically affects its composition.

In developed countries rice milling involves a very sophisticated process. Initially, the paddy rice is cleaned through coarse screens to remove straw, stones and other foreign objects that are larger than the rice kernel. This process is repeated using fine screens in order to remove small weed seeds, sand, stones and other objects smaller than the rice kernels. Stones are separated from the rice in specific gravity tables that separates the product by density. Any metallic particles are removed by magnetic separators. After this cleaning step, the husk is removed by passing the rice through two spinning rubber rollers, which rotate in opposite direction at different speeds (Bond, 2004). Brown rice is obtained after de-hulling. This product can be either eaten as it is, milled into white rice, or processed to obtain different products and by-products. The brown color is due to the presence of bran layers, which are rich in minerals and vitamins. Milled rice, also known as milled white rice, polished rice or polished white rice, is obtained after removing the bran and germ from brown rice.

There are many machines and methods designed for milling rice, but often an abrasive system, followed by frictional and polishing systems are used. During the first step, 95% of the bran is removed in an abrasive whitener, by contact of the grain over an abrasive surface. Subsequently, the bran layers that remain on the grain are removed by friction between the grains using a friction whitener. The degree of milling does not increase linearly with the milling time (Lamberts *et al.,* 2007). A change during the milling process is observed, which is attributed to the different hardness of the bran; the hardness of the bran decreases from outer to inner layers. However, the different endosperm fractions have a similar hardness. The transition from the bran to the endosperm is reached when the degree of milling is approximately 9%.

The color of rice, an important quality parameter, is related to the degree of milling, since the distribution of the pigments is not uniform in the grain. The brightness of the raw kernels and rice flours increases according to the degree of milling, until the bran and the outer endosperm are removed. The bran and outer endosperm contain more red and yellow pigments than the middle and core endosperm. However, these pigments are uniformly distributed in the middle and core endosperm.

Most rice is consumed as a grain. However, rice kernels can be cracked in the field or during drying or milling processes. Often, these cracks lead to the breaking of the kernel, generating broken rice. Rice milling can yield from 4 to 40% broken kernels depending on the incoming rice quality and the milling equipment. Broken kernels are separated from the whole kernels by indent graders, because they tend to get mushy during cooking, thus decreasing the quality of the table rice. Broken kernels can be further separated into various sizes according to their final use (brewing, screening, flour milling). In some countries broken rice is sold as it is, but at lower price than the milled rice. Broken rice is also used for the production of beer, high fructose syrup, flour and high protein flour, starch, maltodextrins, glucose syrup, feed for livestock, spirits, or distilled liquors.

In conclusion, the milling of paddy rice produces milled rice, broken rice, rice bran and hulls and husks. Numerous products with added value have been developed from rice, such as convenience processed rice forms (parboiled, germinated, etc.), rice flour, puffed and crisped rice, breakfast cereals and snacks (Barber and Benedito, 1970; Nguyen and Tran, 2000; Wilkinson and Champagne, 2004).

Grinding

Broken kernels of rice can be ground into flour using three different methods (Yeh, 2004). (1) Wet grinding consists first in soaking the broken kernels in water. After draining, the kernels are ground in the presence of water, in order to reduce the amount of damaged starch. The excess water is removed by drying and the flour is again reground, yielding the wet rice flour. This product is used in the production of different Asian specialities such as Japanese cake, Taiwanese cake, Indian fermented foods, etc. (2) Wet grinding in the presence of 0.3-0.5% NaOH is used for the production of rice starch and rice maltodextrins and syrups. (3) Semi-dry grinding also involves soaking, draining, and grinding without using any excess of water. The semi-dry flour has similar applications to the wet rice flour. Dry grinding is also possible; in this case broken kernels are directly ground to different sizes. Dry rice flour is used for baking, baby foods, extrusion-cooked products and for the production of high-protein flour.

Rice flour properties

Rice varieties can be classified according to their original cultivation area, grain size and amylose content. Indica rice has been grown in India, Bangladesh, Vietnam, Thailand, Pakistan, etc., while Japonica rice was cultivated in Japan, Korea as well as northern and central regions of China. Based on the grain size, rice can be classified as long (longer than 6.6 mm), medium (between 5.5 and 6.6 mm), or short (shorter than 5.5 mm). The amylose content differs between waxy (less than 1% amylose) and

non-waxy (higher than 10% amylose) rice. Rice is mainly consumed as polished rice and, thus, primary differences among different types of rice rely on their cooking characteristics, although they also differ in their physico-chemical properties (Vasudeva *et al.,* 2000). Rice flour can be obtained from complete grains, but it is usually produced from the kernels broken during the milling process because their cost is lower than that of the whole milled kernels. Usually rice flours have the same chemical composition as parent-milled kernels. The characteristics of the rice flours are governed by inherent cultivar's variations, environmental variation, the grinding methods and their previous treatments.

Rice flours mainly differ in the amylose content, which determines the gelatinization temperature, and in general the pasting behaviour and viscoelastic properties (Fan and Marks, 1998; Singh *et al.,* 2000; Meadows, 2002; Saif *et al.,* 2003; Rosell and Gómez, 2006). Analysis of the pasting behaviour is a useful method to characterize the properties of the rice flour. Although the amylograph was the equipment traditionally used, in recent years, it has been replaced by the rapid viscoanalyzer (RVA), since the latter allows a better understanding of the pasting properties with high precision, sensitivity and rapidity (Meadows, 2002; Gujral *et al,* 2003a). The pasting properties of rice flours greatly depend on the cultivars; in fact, rice breeders frequently use the RVA as an index of rice quality. Rice flours from Bomba and Thaibonet cultivars show higher pasting temperatures, which correspond to high gelatinization temperatures and lower peak viscosities, resulting from their high amylose content. These properties are typically attributed to long grains (Rosell and Gómez, 2006; Rosell and Collar, 2007). However, Bomba has very short grains that during cooking behave as long grains. Bomba rice grains show a low viscosity breakdown during high temperature holding cycles and a marked increase in viscosity during cooling that corresponds to a tendency to retrograde. Therefore, grain length alone can not be used to represent the pasting properties of a rice. In contrast, Bahia

and Senia have higher peak viscosities and lower pasting temperatures, and both show similar behaviour during heating and cooling.

Near-infrared spectroscopy is a rapid technique for determining the protein and amylose content (Miryeong *et al.,* 2004). A new piece of equipment that has just appeared in the market is the Mixolab. The Mixolab allows the mixing and pasting properties of the flours (i.e. flour behaviour under mechanical and thermal constrains) to be determined (Bonet *et al.,* 2006; Rosell *et al.,* 2007). From the plot obtained, it is possible to extrapolate useful information. The first part of the curve, before the heating cycle starts, allows the water absorption of the flour to be determined. The target of a torque of 1.1 Nm approximately corresponds to 500 BU obtained with the Brabender Farinograph. In the second part of the curve, similar results can be obtained as those commonly originating from the RVA. However, the Mixolab works with dough systems, whereas RVA analysis is performed on suspensions. The different slopes of the curve during the assay are related to different properties of the flour: speed of the weakening of the protein network due to heating (α); gelatinization rate (β); and enzymatic degradation speed (γ). For example, Mixolab allowed the effect of water addition on rice flour properties to be determined (Fig. 2; Tables 2 and 3). As the water addition increased, a decrease in the dough consistency was detected, in agreement with the dilution effect of the dough. This difference was higher during the mixing step (first part of the curve), where the proteins play the main role when dough is affected by a mechanical constraint (Rosell *et al.,* 2007). However, during the heating and cooling cycles, the differences between samples with different water content decreased. The amount of water present in the system, although limited for starch gelatinization, was sufficient to gelatinize a large amount of starch.

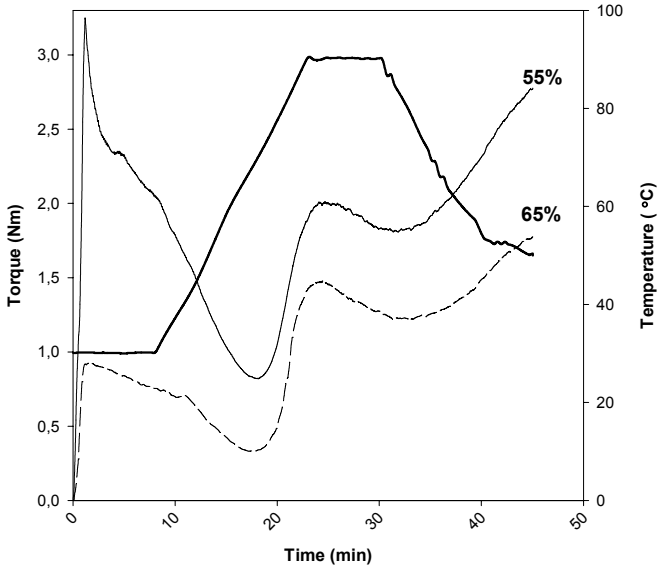

Figure 2. Mixolab analysis of rice dough behavior during mixing, heating and cooling when different amounts of water are added. Temperature: thick line. Numbers are referred to the amount of water (expressed in percentage, flour basis) added for dough mixing.

In order to achieve a suitable consistency for breadmaking, rice flour doughs require a very high hydration compared with wheat flour doughs. The addition of large quantities of water leads to considerable improvement of the dough behaviour during mixing (i.e., higher stability). During heating-cooling cycles, higher hydrated doughs result in lower peak torque (related to the starch gelatinization) and also lower final torque at the end of the cooling, due to the starch dilution effect. More hydrated rice doughs also have lower setback (related to the amylose tendency to retrograde), due to the amylose dilution, whereas the rate of starch gelatinization (α) and enzymatic degradation speed (γ) increase because of the large amount of water available.

Table 2. Effect of the addition of different amounts of water on the rice flour behaviour studied by the Mixolab device.

Water level (%, flour basis)	Development time (min)	Maximum consistency (Nm)	Amplitude (Nm)	Stability (min)	Minimum torque (Nm)	Peak torque (Nm)	Torque at the end of heating (Nm)	Final torque (Nm)
55.0	1.18	3.25	0.276	0.9	0.82	2.01	1.81	2.78
65.0	1.62	0.93	0.051	1.12	0.33	1.48	1.22	1.78

Table 3. Derived parameters obtained from the Mixolab curves of rice flour doughs with different hydration.

Water level (%, flour basis)	Derived parameters				
	Cooking stability	Setback (Nm)	α (Nm/min)	β (Nm/min)	γ (Nm/min)
55.0	0.90	0.97	-0.156	0.340	-0.018
65.0	0.82	0.56	-0.090	0.384	-0.042

Since the pasting properties influence the behaviour during baking, careful selection of the rice cultivars used in the grinding is recommended when the flour is destined for bakery. In general, the long-grain varieties have higher amylose content, higher gelatinization temperatures, and a greater tendency to retrograde than medium- or short-grain varieties. Rice flours can be also obtained from waxy rice varieties. These waxy varieties have an amylose content lower than 1% and low gelatinization temperatures (61-62ºC). Even if their properties are not appropriated for baking, these flours can be used as minor ingredients (Bean *et al.,* 1984).

Environmental variation also plays a significant role in determining the pasting properties of rice flour. In fact, Minh-Chau-Dang and Copeland (2004) studied the effect of growth season and location on the pasting properties of three different rice cultivars (Doongara, Langi and Kyeena). Genotype, growth season and growth location all affected the pasting behaviour of the rice flour. The amylose content of individual cultivars was significantly higher in the coolest growing season, resulting in RVA traces with lower peak viscosities and higher setbacks than samples with lower amylose contents. When the same rice cultivar was grown in different locations in the same season, no significant differences could be detected in the chemical composition of the resulting flours. However, significant differences were found in the pasting behaviour, indicating that the environment influences the pasting behaviour of rice flour. In conclusion, it was found that the pasting behaviour of rice flour is related to genotype and influenced by environmental factors that result in minor changes in the grains that are not detected by chemical analyses.

The physical properties of the rice flour are also affected by the time elapsed between harvest and milling, as well as by the temperature used in the drying process prior to storage. The influence of rice moisture content at harvesting on the rice flour properties was investigated for long- and medium-grain rice (Linfeng-Wang *et al*. 2004). Peak viscosity of the flour, an indicator of rice functionality and performance,

increased as the rice harvest moisture content decreased, although the rate of increase was influenced by the rice cultivar and growing location. Moreover, duration and temperature of storage were found to significantly affect the enthalpies and temperature of gelatinization and retrogradation of rice flour (Fan and Marks, 1999). Recently, Zhou *et al*. (2003) observed that the time and temperature of rice storage also influence the RVA pasting curves of rice flours from different cultivars. In this work, a change in the protein profile was observed, with a positive correlation between increase in the amount of high molecular weight peptides and storage time. In fact, the changes in the structure and properties of oryzenin, rather than the starch, are responsible for the modification of the rice physical properties associated with storage (Teo *et al.,* 2000; Patindol *et al.,* 2003).

The conditions of the milling process are very important, since the number of drying steps and temperature during tempering affect the rough rice quality (Correa *et al.,* 2007). Two- or three-step drying reduces the percentage of fissured kernels compared with one-step drying (Aquerreta *et al.,* 2007). The tempering at high temperature (60ºC) also reduced the percentage of fissured kernels independently of the number of drying steps.

The grinding method employed will also affect the functional properties of the rice flour. The method and type of mill determine the particle size of the rice flour and also the amount of starch damage. Nishita and Bean (1982) reported a comparative study about the properties of rice flours obtained with different mills. Roller mills led to rice flours with medium granulometry that showed good performance in the bakery. In contrast, the burr mills yielded excessive coarse flour that produced low-quality breads. The use of hammer mills led to finer particles with high levels of damaged starch that are not adequate for breadmaking, but could be used in cake production. With regard to pasting properties, the flours with greater particle size showed lower peak viscosity and final viscosity at 50ºC (low tendency to retrograde), while the flours

with medium or small particle size did not show any significant changes in their properties.

The enthalpy values obtained by differential scanning calorimetry (DSC) are indicative of the degree of the starch damage occurring during grinding. Lower enthalpy values are related to higher damage of the starch. Thermally stimulated luminescence (TSL) can also be used as thermal analysis technique, since the observed TSL is due to permanent phase change in the specimens (Murthy *et al.,* 2007). Irradiated rice flour can be detected by this technique since the generation of electron/hole traps result in an increase in TSL peak intensity.

Another luminescence technique is photoluminescence (PL) (Katsumata *et al.,* 2005). Peak intensity of PL varies depending on the variety and source of the rice. Two-dimensional (2D) images of photoluminescence allow blended rice from different species, contamination and foreign objects to be detected, making this technique potentially useful for non-destructive and quick evaluation of rice products for quality control purposes (Katsumata *et al.,* 2005).

The rheological properties of the flour are also influenced by the temperature, moisture and lipids content. Dautant *et al*. (2007) found that at constant moisture content, the viscosity decreases when the temperature increases and also when the shear rate increases, regardless of the temperature applied. This decrease in the viscosity with the increase in the shear rate demonstrates the pseudoplastic nature of the material. An increase in the moisture or in the lipid content (up to 5%) also results in a decrease in viscosity. During extrusion processes rice flour is cooked. Therefore, it is important to take into account the viscous behavior of the rice flour in order to establish the best processing conditions to be applied in the food industry, since this will affect the quality of the end products.

Rice flours are usually obtained from polished or milled kernels, although sometimes brown rice is employed for grinding. Flours obtained from brown kernels have a 13- 17°C higher temperature of gelatinization and around 40% greater gelatinization enthalpy than their milled counterparts (Normand and Marshall, 1989). Flours from brown rice contain high amount of fiber and vitamins, predominant in the outer layers of the kernel. These compounds confer special organoleptic properties (color, texture and taste) on the baked products. However, brown rice flours have a very short shelf-life. This is due to the presence of active lipase and lipooxygenase, and thus to the release of free fatty acids, which start going rancid, imparting a bitter taste to the products. The stability of these flours can be increased by reducing the temperature and humidity during storage, or using inert atmospheres; however, these modifications affect the cost of the product. As an alternative, brown rice flour can be obtained by adding milled bran at appropriate levels to already ground rice. In this case, the bran can be chemically or physically treated previously to ensure its stability and to extend the shelf-life (Champagne *et al.,* 1991; Champagne and Grimm, 1995). A different approach is to remove the bran fat. Rice bran, defatted or not, can also be used as a source of fiber and vitamins in wheat-based products (Lima *et al.,* 2002).

Rice flours can also be obtained from cooked rice, a process which modifies the rheological behavior in steady and dynamic shears (Chun and Yoo 2004). Processes such as parboiling the kernels before milling can modify the physico-chemical characteristics of rice. In the parboiling process, paddy rice is soaked and steamed under pressure in order to gelatinize the starch within the kernel. After cooling, slow drying reduces the formation of cracks. The conversion of the starch from a crystalline to an amorphous state favors the migration of nutrients from the bran layer to the starchy endosperm. Parboiled rice thus has higher levels of nutrients (vitamins and minerals) and different sensory properties. Flours obtained from parboiled rice produce soft and sticky doughs, due to the low water retention capacity and the high

susceptibility to amylase attack. Therefore, these flours are not suitable for breadmaking, but they can be used in small concentrations for cake production, where the short process time reduces the activity of the amylases. Pre-gelatinized rice flour can be obtained by extrusion, puffing, or roasting. All these treatments negatively affect the rheological properties of the rice flour, leading to sticky doughs and low-volume breads (Bean and Nishita, 1985).

PRODUCTION AND CHARACTERIZATION OF GLUTEN-FREE CEREAL PRODUCTS BASED ON RICE

Rice is mainly consumed as milled rice, although fiber and minerals are lost during the milling process. As a food ingredient, rice confers creaminess, crunchiness, and firmness to the final product. Beside the common use as table rice, rice can be used for the production of beer, baby foods, breakfast cereals, snacks, confections, desserts, as well as bakery products. The increased use of rice in food processing is the result of increasing consumer demands for healthier and more convenient products, as well as a growing interest in ethnic products. Moreover, rice-based products represent the solution for consumers with allergenic problems. In addition, husks, hulls and bran are used as energy sources, fillers for polymeric composite, and raw materials for the production of nutraceuticals and protein concentrates.

Dry rice breakfast cereals include rice flakes, oven, gun or extruder-puffed rice, shredded-rice cereals, and multigrain cereals. These products are prepared by pressure-cooking in the presence of sugar, salt, flavorings and sufficient water. Rice flakes are prepared in a similar way to wheat and corn flakes: the rice is cooked and coated with nutritious ingredients (skimmed milk) and then partially dried, tempered and passed through flaking rolls before toasting in an oven (Wilkinson and Champagne, 2004).

Rice snacks include granola, breakfast, and energy bars (Juliano and Hicks, 1996). Some snacks are designed as functional foods (e.g., they can help to reduce cholesterol levels). A number of these products are aimed at children, women, and other specific groups. Rice flour is used in many Asian snacks, since it is the most cultivated cereal in these countries. Rice noodles are obtained by extrusion and rice flour with a high amylase content is usually used. The process consists of partial cooking of the dough, kneading and forming, final cooking, and drying. Rice noodles are consumed as main foods, soups, or snacks. According to the production process, cakes can be divided into pastry, unleavened, dry or fermented cakes (Rosell and Gómez, 2006). Finally, crackers can be obtained using non-waxy rice (i.e. senbei) or waxy rice (i.e. arare). Rice flour is extensively used for the production of infant food formulas due to its digestibility and hypoallergenic properties. A partial acid or enzymatic (using starch-hydrolyzing enzymes) hydrolysis of the rice flour is applied in order to increase the concentration of free sugars, contributing to the sweet taste and consistency (Cantoni, 1967).

Rice flour is increasingly used in baking as a substitute for wheat for the preparation of products intended for wheat-intolerant or coeliac patients. It is the most suitable cereal grain flour for the production of gluten-free products due to its bland taste, white color, digestibility, and hypoallergenic properties (Neumann and Bruemmer, 1997). In addition, other attributes such as the low content of protein and sodium, the low levels of prolamins and the presence of easily digested carbohydrates make rice the best cereal for patients suffering from allergies. However, in spite of the numerous advantages of rice flour, rice proteins have relatively poor functional properties for food processing. Due to their hydrophobic nature, rice proteins are insoluble and unable to form the viscoelastic dough necessary to hold the carbon dioxide produced during proofing of yeast-leavened bread-like products. The low content of prolamins in rice flours results in the lack of

formation of a protein network when rice flour is kneaded with water. As a consequence, the carbon dioxide produced during fermentation cannot be retained, leading to a product with low specific volume and a very compact crumb (Fig. 3) which does not resemble the soft and open structure of common wheat bread (He and Hoseney, 1991).

To improve the quality of bread, structuring agents, such as xanthan gum and carboxymethylcellulose (CMC), are commonly added to gluten-free bread formulations (Kulp *et al.,* 1974). Recently, pectin, CMC, agarose, xanthan, or oat β-glucans were used in gluten-free formulations based on rice flour, corn starch and sodium caseinate (Lazaridou *et al.,* 2007). With the exception of xanthan gum, the presence of these hydrocolloids resulted in breads with higher volume. Finally, breads supplemented with 2% CMC received the best score on sensory testing. Among the cellulose derivatives, hydroxypropylmethylcellulose (HPMC) seems to be a suitable gluten substitute in rice bread formula due to its gas retention capacity and its properties as a crumb-structuring agent (Nishita *et al.,* 1976; Ylimaki *et al.,* 1988; Gujral *et al.,* 2003a). Upon addition of HPMC, the consistency and rheological properties of rice doughs closely resemble those of wheat doughs (Fig. 4) (Sivaramakrishnan *et al.,* 2004). The presence of 4% (flour basis) HPMC leads to a significant increase in bread volume and loaf structure (Fig. 5). Other gums such as locust bean gum, guar gum, carrageenan, xanthan gum and agar have been tested as gluten replacers in rice bread (Kang *et al.,* 1997, Cato *et al.,* 2004, Lazaridou *et al.,* 2007). In general, the volume of rice breads increases with the addition of hydrocolloids except for xanthan; however, increasing the level of hydrocolloids from 1% to 2% results in a decrease in loaf volume, except for pectin. High values of crumb porosity are obtained when 1% CMC and β-glucans or 2% pectin are added, whereas high crumb elasticity is induced by CMC or pectin addition.

Figure 3. Bread obtained from rice flour without any additive (control) (Photo by Cristina Marco).

Figure 4. Bread obtained from rice flour in the presence of 4% HPMC (Photo by Cristina Marco).

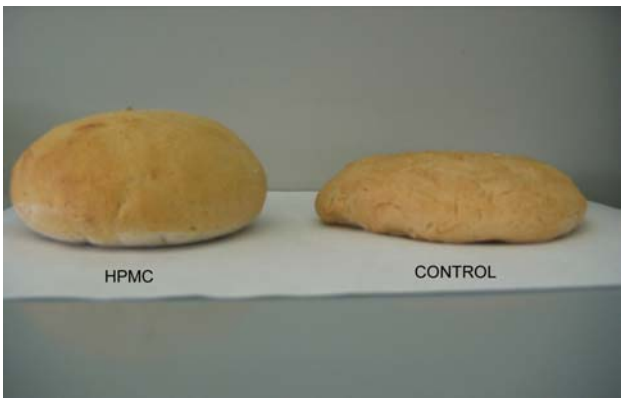

Figure 5. Bread obtained from rice flour without additive (control) and in the presence of 4% (flour basis) hydroxypropylmethylcellulose (HPMC) (Photo by Cristina Marco).

Addition of hydrocolloids has allowed the production of rice breads with a loaf specific volume comparable to that of wheat bread, however sensory appearance and crumb texture are still poor. Improvement of the crumb texture has recently been achieved with the addition of vegetable seed oil (Gujral *et al.,* 2003a). In contrast to wheat flour, rice flour is not responsive to the presence of dough conditioners or enzymes (Nishita *et al.,* 1976), probably due to the hydrophobic nature of rice proteins. However, recent studies have shown the usefulness of some enzymes in the making of rice-based products. For example, addition of cyclodextrin glycosyl transferase (CGTase) led to rice loaves with very soft crumbs (Gujral *et al.,* 2003a, 2003b). This enzyme acts as an α-amylase that can use the hydrolysis products to produce cyclodextrins that can form complexes with a variety of solid, liquid and gaseous compounds. The improving effect of CGTase in rice breadmaking was due to the formation of complexes between lipids and proteins with the cyclodextrins. The addition of CGTase also helped to extend the shelf-life of rice bread, acting as anti-staling agent through its hydrolyzing and cyclizing activity (Gujral *et al.,* 2003b). Another enzyme with anti-staling activity is α-amylase. This is an endo-enzyme that randomly hydrolyzes the α-1,4 glucosidic linkages in polysaccharides, resulting in short chains that can be fermented by yeast. α-Amylase of intermediate thermostability has been shown to improve the shelf life of gluten-free bread, increasing the crumb softness and elasticity of bread (Novozymes, 2004). Laccase (p-diphenol oxygen oxidoreductase) is an oxidative enzyme that catalyses the oxidative gelation of feruloylated arabinoxylans by dimerization of their ferulic esters (Figueroa-Espinoza *et al.,* 1998; Labat *et al.,* 2001). The addition of small levels of laccase (1.5 U/g flour) improved the rice-bread specific volume, but increasing the enzyme levels showed detrimental effects, particularly regarding crumb hardness (Gujral and Rosell, unpublished results).

Other enzymes with good potential in rice bread formulation are glucose oxidase and transglutaminase (Gujral and Rosell, 2004a, 2004b). These enzymes promote the formation of a protein network by catalysing inter- and intra-molecular crosslinks between the rice proteins. However, the protein network catalyzed by enzymatic treatment of rice proteins does not completely meet the gluten functionality, and thus a reduced amount of hydrocolloids is still needed (Gujral and Rosell, 2004a and 2004b). When considering transglutaminase applications, addition of an external source of proteins has been suggested in order to increase the amount of lysine residues, which are the limiting factor of the cross-linking reaction (Moore *et al.,* 2006). Exogenous protein sources such as soybean flour, skim milk powder, or egg powder were therefore added (12.5% composite flour basis) to a gluten-free bread formulation (containing rice flour, potato starch, corn flour, xanthan gum) in the presence of increasing levels of transglutaminase. Confocal laser scanning micrographs of the bread crumbs confirmed the cross-linking of dairy proteins, although high amounts of transglutaminase (10 U/g of protein) were needed, probably because of the thermodynamic incompatibility between polar and apolar surfaces of milk proteins (Moore *et al.,* 2006). The compatibility between rice flour proteins and different protein isolates (pea, soybean, egg albumen and whey proteins) in the cross-linking reaction catalyzed by transglutaminase has been evaluated studying the rice dough behaviour subjected to small deformations (Marco and Rosell, 2007). The elastic modulus recorded in the oscillatory tests was significantly affected by both the protein isolates and the transglutaminase. The extent of the effect was dependent on the protein source; pea and soybean proteins increased the elastic modulus, whereas egg albumen and whey protein decreased it.

A deeper evaluation of the cross-linking in the presence of soybean proteins, by using different electrophoretic techniques, indicated that the main protein fractions involved in these interactions were both β-conglycinin and glycinin of soybean as well

as the glutelins of rice flour, although albumins and globulins were also cross-linked (Marco, Pérez, León, Rosell, results unpublished). The interaction between rice and soybean proteins was intensified by the formation of new intermolecular covalent bonds catalyzed by transglutaminase, and also by the indirect formation of disulfide bonds between proteins. Concerning the pea proteins, main protein fractions involved in the interactions were the albumins and globulins from the pea protein isolate and rice flour, but also the glutelins were cross-linked (Marco *et al.,* 2007). In conclusion, the studies carried out with different protein sources have shown that their combination with network-forming enzymes has great potential for enhancing the structure of gluten-free products. Recently, chemical modifications of rice flour allowed the production of rice-bread with similar texture characteristics to those of wheat bread (Nabeshima and El-Dash, 2004).

Some bread specialties have been adapted to obtain gluten-free products addressed to people suffering from gluten intolerance. This is the case of chapatti, an unleavened bread made from whole wheat in India. The use of different hydrocolloids (HPMC, guar gum, xanthan gum, or locust bean gum) and α-amylase in the formulation of rice flour chapattis improved the texture by keeping the extensibility during storage (Gujral *et al.,* 2004c). In addition, hydrocolloids and α-amylase delayed the amylopectin retrogradation, keeping the freshness of the chapattis during longer period.

A different approach to the production of gluten-free bread is to use rice flour blended with other flours and different starches (Gallagher *et al.,* 2004). Complex formulation including corn starch, brown rice, soy and buckwheat flour have been proposed (Moore *et al.,* 2004). Using these recipes, breads were brittle after 2 days of storage, although this effect was reduced when dairy products such as skimmed milk powder were included in the formulation. In addition, a combination of rice flour (45%)

with corn (35%) and cassava (20%) starches gave a good gluten-free bread with uniform and well distributed cells over the crumb as well as a pleasant flavor and appearance (Lopez *et al.,* 2004). Gluten-free breads of good quality were also obtained using small amounts of rice flour (about 17.2%) and using corn starch (74.2%) and cassava starch (8.6%) (Sanchez *et al.,* 2002). Finally, blends of buckwheat and rice flours in the presence of hydrogenated vegetable fat also have the potential to give gluten-free breads with good sensory attributes (Moreira *et al.,* 2004).

FUTURE TRENDS

Rice is an important source of energy, providing 26% of the total energy intake in developing countries, although it provides only 4% of the total energy intake in the developed world. In developing countries, rice supplies 20% of the dietary protein intake, but because of its incomplete amino acid profile and the limited levels of micronutrients (especially in milled rice), the use of rice as staple food may lead to malnutrition. Patients with celiac disease already tend to have malnutrition, since the immunological reaction induced by gluten ingestion produces damages to the mucous of the small intestine, reducing its nutrient absorption capacity. In addition, most gluten-free products are low in micronutrients, which increases the risk of deficiencies. In order to improve the nutritional quality of gluten-free products based on rice, other protein sources can be added. Dairy and soybean proteins are the most used. Legume proteins are a good supplement for cereal based foods, since both legume and cereal proteins are complementary in essential amino acids. Nowadays, different techniques for rice fortification have been developed in order to add essential vitamins and minerals to the grain (Nunes *et al.,* 1991; Hoffpauer and Wright, 1994; Rosell, 2004). Alternatively, specific minerals can be added to the products during the manufacturing process. For example, Kiskini *et al*. (2007) obtained gluten-free bread

fortified with iron (incorporated as ferric pyrophosphate) that presented good sensory and nutritional characteristics. However, these compounds might affect the sensory quality of the products, and therefore particular attention has to be given to the form and amount of added compounds.

Rice is mainly consumed as milled rice, although brown rice has better nutritional value. Brown rice is obtained after dehulling, and the brown color is due to the presence of bran layers, which are rich in minerals and vitamins. Brown rice contains more nutritional components than the ordinary milled rice grains (e.g. dietary fibers, phytic acids, E and B vitamins, and γ-aminobutyric acid (GABA)). All these compounds are present in the bran layers and germ that are removed during polishing or milling (Champagne *et al.,* 1991, 2004; Champagne and Grimm 1995). Despite the nutritional benefits linked to its consumption, brown rice is not considered suitable for table rice because it has to be cooked in a pressure rice cooker, and also because of its dark appearance and hard texture. Moreover, when the husk is removed from rice, the bran layer starts going rancid, contributing to the bitter taste of the brown rice. This is why brown rice is mainly used for fermentation purposes, or in materials for food processing.

The use of germination in grains started some decades ago, mainly applied to wheat and soybean (Finney, 1978; Tkachuk, 1979). Germinated brown rice arose following research into the development of new value-added products from rice. In 1994, Saikusa *et al*. found that GABA levels increased significantly when brown rice was soaked in water at 40°C for 8-24 hours. An increase in dietary GABA intake has been found to lower blood pressure, improve sleep and the autonomic disorder associated with the menopausal or presenile period, and can even suppress liver damage (Okada *et al.,* 2000; Tadashi *et al.,* 2000; Jeon *et al.,* 2003). In Japan, germinated brown rice was launched on the market in 1995. Since then, it has increased in popularity within the Japanese population, and numerous industries have

emerged in Japan related to its production. During the last decade, 49 items related to germinated brown rice have been patented. The basic procedure for obtaining pre-germinated brown rice consists in the selection of good brown rice, which then is soaked for around 20 hours at 30 to 40°C. This product is washed slightly before cooking, and is marketed either dry or wet (i.e. 15 or 30% moisture, respectively). During the germination process, saccharification softens the endosperm and dormant enzymes are activated, leading to an increase in the amount of digestible compounds (Manna *et al.,* 1995). In addition, the mineral content changes, resulting in an increase of GABA, free amino acids, dietary fiber, inositols, ferulic acid, phytic acid, tocotrienols, magnesium, potassium, zinc, γ-oryzanol and prolylendopeptidase inhibitor (Kayahara and Tsukahara, 2000; Ohisa *et al.,* 2003; Ohtsubo *et al.,* 2005). Germinated brown rice can be cooked in an ordinary rice cooker, giving a soft product with easier chewiness. Moreover, it can be used as a raw material in the production of various foods, including germinated brown rice balls, soup, bread, doughnuts, cookies and rice burger (Ito and Ishikawa, 2004).

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Objetivos y Plan de Trabajo

OBJETIVOS

El objetivo de la tesis doctoral es la obtención de productos panarios libres de gluten enriquecidos en proteínas, mediante el desarrollo de miméticos de gluten a través de la generación de enlaces cruzados entre las cadenas proteicas catalizados enzimáticamente.

En dicho objetivo general se incluyen los siguientes objetivos particulares:

- Obtener redes proteicas mediante la generación de enlaces cruzados entre las cadenas proteicas.
- Extender el uso de la harina de arroz a la elaboración de productos fermentados de panadería libres de gluten.
- Analizar y potenciar la interacción entre las proteínas de la harina de arroz y otras fuentes proteicas.
- Desarrollar nuevos productos panarios fermentados derivados de cereales libres de gluten mediante la aplicación de materias proteicas con mayor valor nutritivo.

PLAN DE TRABAJO

Para la consecución de los objetivos anteriormente propuestos se propone el siguiente plan de trabajo:

- Selección de los aislados proteicos más adecuados para su utilización en la elaboración de productos fermentados libres de gluten. Para ello se estudiará el efecto de la adición de proteínas de diversas fuentes (soja, guisante, albumen de huevo, suero lácteo) a la harina de arroz, así como el efecto que produce la transglutaminasa en estas proteínas.

- Estudio del tipo de interacción entre las proteínas debido al tratamiento enzimático con transglutaminasa e identificación de las fracciones proteicas involucradas en el entrecruzamiento. Para ello se realizarán estudios bioquímicos de aislamiento y caracterización de las fracciones proteicas.

- Aplicación de un diseño experimental para evaluar el efecto de la adición de diferentes dosis de las proteínas seleccionadas y de TG en las propiedades reológicas y funcionales de las masas.

- Los resultados obtenidos en los apartados anteriores se extrapolarán a un sistema real de producción de productos fermentados dirigidos a la población con celiasis. En los productos finales obtenidos se evaluará el volumen específico, textura, color y microestructura.

Capítulo 1

Effect of different protein isolates and transglutaminase on rice flour properties.

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Effect of different protein isolates and transglutaminase on rice flour properties

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Abstract

The effect of the addition of different protein isolates (pea, soybean, egg albumen and whey proteins) on the viscosimetric and rheological properties of the rice flour dough, and the development of a protein network through the use of microbial transglutaminase (TG) were evaluated. Protein isolates significantly (p<0.05) modified the gelatinization and gelling behaviour of the rice starch determined in the rapid viscoanalyser (RVA). Pea, soybean and whey proteins significantly (p<0.05) decreased the final viscosity in addition, whey protein also promoted a significant decrease (27.3%) in the peak viscosity. The elastic modulus value (G´) recorded in the oscillatory tests was significantly (p<0.01) affected by both the protein isolates and the TG. The extent of the effect was dependent on the protein source; pea and soybean increased this parameter, whereas egg albumen and whey protein drastically decreased it. A modification in the emulsifying properties was also observed by the addition of protein isolates and the TG treatment. The decrease in the amount of free amino groups after TG treatment confirmed the protein crosslinking catalysed by TG. Therefore, the use of protein isolates and TG broads the applications of rice flour in the bakery industry, and brings about an increase in the protein content with the subsequent nutritional improvement of the resulting products.

Keywords: rice flour, protein isolates, transglutaminase, rice-protein blends properties, rheology.

INTRODUCTION

Rice flour has soft taste, colourless, hypoallergenic properties, low levels of sodium and easy digestible carbohydrates. Because of these properties, rice flour is the most suitable cereal to make gluten free products (Gujral, Guardiola, Carbonell & Rosell, 2003a; Gujral, Haros & Rosell, 2003b; Gujral & Rosell, 2004a, 2004b; Lopez, Pereira & Junqueira, 2004). However, gluten free cereals like rice do not meet the requirements necessary for processing fermented food products because their proteins cannot develop a viscoelastic network like gluten, enable to retain the CO_2 produced during fermentation process. Because of this, different strengtheners agents mainly included in the category of gums and starches have been proposed (Kang, Choi and Choi, 1997; Nishita, Roberts & Bean, 1976).

The addition of starches, gums and hydrocolloids to gluten free products provide the necessary network for obtaining fermented bakery products. The effect of several hydrocolloids -hydroxypropylmethylcellulose (HPMC), locust bean gum, guar gum, carrageenan, xanthan gum, and agar- on rice bread has been already tested (Kang et al., 1997). All of them gave successful formation of rice bread, but the addition of HPMC yielded rice breads with the optimum volume expansion, up to 4% of HPMC might be added for obtaining rice dough with similar rheological properties to wheat flour dough and good rice bread specific volume (Gujral et al., 2003a; Gujral & Rosell, 2004a, 2004b; McCarthy, Gallagher, Gormley, Schober & Arendt, 2005; Sivaramakrishnan, Senge & Chattopadhyay, 2004). Starches from corn, cassava and potato are usually found in gluten free bread formulations (Lopez et al., 2004; McCarthy et al., 2005; Sánchez, Osella & de la Torre, 2002; Schober, Messerschmidt, Bean, Park & Arendt, 2005). Diverse flours are mixed with a high proportion of different starches affecting the crumb bread structure and also the staling process due to the different starch crystallites formed during storage (Osella, Sánchez, Carrara, de la Torre & Buera, 2005). Nevertheless, gluten free products

resulting from mixing starches and gums with a proportion of gluten free cereals have a very low protein content and are lysine defficient. Proteins from different sources (soybean, pea, egg albumen, whey) can be added in order to increase the nutritional value of gluten free products (FAO, 1981). With that purpose, dairy ingredients have been used in the production of gluten free products resulting in nutritional benefits and improved volume, appearance and sensory aspects of the loaves (Gallagher, Gormley & Arendt, 2003a; Gallagher, Kunkel, Gormley & Arendt, 2003b; Sánchez, Osella & de la Torre, 2004). Nevertheless, the addition of increasing amounts of skim milk powder induced a decrease in loaf height (Schober et al, 2005). Soy and egg proteins have also been added to gluten free products resulting also in better loaf volume (Gallagher et al, 2003a; Kobylanski, Perez & Pilosof, 2004; Ribotta et al., 2004). However, although initially the aim of addition of proteins was to increase the nutritional value of gluten free products, lately, it has been reported that the formation of a continuous protein phase is critical for obtaining an improvement in the quality of gluten-free bread (Moore, Schober, Dockery & Arendt, 2004). Therefore, the selection of the protein source with the appropriate functionality seems to play an important role in the breadmaking process of gluten free breads. In addition, if a network-like structure could be created with those proteins and the gluten free cereal proteins, that could hold the dough structure during proofing, resembling the gluten network obtained in wheat bread dough.

Protein functionality can be modified by forming intramolecular or intermolecular crosslinks (Gerrard, 2002; Jong & Koppelman, 2002). Transglutaminase (TG) catalyses the reaction between an ε-amino group on protein-bound lysine residues and a γ-carboxyamide group on protein-bound glutamine residues, leading to the covalent crosslinking of the proteins. This is the most dominant reaction, but TG also catalyses two other reactions: in the presence of primary amines, TG crosslinks the amine to a γ-carboxyamide group on protein-bound glutamine residues. In the

absence of amine substrates, TG catalyses the hydrolysis of the γ-carboxyamide group of glutamine, resulting in deamidation. This enzyme has been used for improving the baking quality of the weak flours (Autio et al., 2005), bringing about an improvement in the texture of the loaves (Gerrard et al., 1998). In addition, some improvement in the rice protein functionality was observed when they were crosslinked with TG (Gujral & Rosell, 2004a), which allowed decreasing the levels of HPMC needed to obtain acceptable rice bread.

The aim of the present study was to screen the effect of the addition of different protein isolates on the properties of the rice flour dough, and the contribution of the transglutaminase activity to the thermal viscosimetric profile and viscoelastic behaviour of the rice flour-protein blends.

MATERIALS AND METHODS

Commercial rice flour, from Harinera Belenguer SA (Valencia, Spain), had moisture, protein, fat and ash contents of 13.4, 7.5, 0.9 and 0.6%, respectively. Protein isolates (pea, soybean, egg albumen and whey) were from Trades SA (Barcelona, Spain). Microbial transglutaminase (100 units/g) was from Apliena, SA (Terrasa, Barcelona, Spain). Composition of the different protein isolates was determined following the ICC-Standard methods (2004) (Table 1). All reagents were of analytical grade.

Pasting properties

Pasting properties were determined using a Rapid Visco Analyser (RVA) (Newport Scientific model 4-SA, Warriewood, Australia) by following the AACC Approved Method No 61-02 (AACC, 1995). RVA analyses were performed on rice flour mixed with 5% (w/w, flour-protein blend basis) of the different protein isolates. When TG was present, 1% (w/w, flour-protein blend basis) of TG was mixed with the rest of the

Table 1. Proximate composition of the protein isolates tested.

	Percentage (%)				
Proteins	Moisture content	Protein	Lipid	Ash	Carbohydrates [a]
pea	6.7	79.2	0.9	4.2	9.1
soybean	6.9	80.8	0.2	3.6	8.6
egg albumen	7.6	88.8	0.2	3.5	0.0
whey	8.9	84.2	0.2	2.2	4.5

[a] Calculated by difference.

powdered ingredients. Viscosity was registered during a heating-cooling cycle: heating from 50 to 95 ºC in 288 s at a rate of 9.4 ºC/min, holding at 95 ºC for 150 s and cooling to 50 ºC in 228 s; the paddle speed was 960 rpm for 10 s and then decreased to 160 rpm for the rest of the cycle. The plots of the samples containing the different protein isolates are showed in Fig. 1. The parameters determined were: peak viscosity, final viscosity, breakdown and setback (difference between final viscosity and peak viscosity).

Rice dough preparation

The dough was made in a 50g bowl Farinograph (Brabender, Germany), mixing 50 g of rice flour with 90% of water (flour basis, corrected to 14% moisture basis) at a paddle speed of 90 rpm for 15 min. When protein isolates were present, rice flour was replaced by 5% (w/w, flour-protein blend basis) protein isolate. The effect of TG was studied by adding 1% (w/w, flour-protein blend basis) TG to the solids. Rice dough was used for the dynamic oscillatory test and the rest of the dough was frozen and freeze-dried. Dry rice dough was milled with a refrigerated micro-hammer mill resulting in a powder with a particle size not exceeding 250 μm.

Oscillatory measurements

Dynamic rheological measurements of the dough were determined on a controlled stress rheometer (Rheostress 1, Termo Haake, Germany). The measuring system consisted of parallel plate geometry (60 mm diameter, 1 mm gap). The rice dough was placed between the plates within 1 h after mixing and the test started after 5 min resting. The rim of the sample was coated with Vaseline oil in order to prevent evaporation during the measurements. Measurements were performed at 30 ºC (Gujral & Rosell, 2004a). Stress sweeps at 1 Hz frequency were carried out to determine the linear viscoelastic zone. Frequency sweep tests were performed from 0.01 to 10 Hz to determine the storage modulus (G´), loss modulus (G´´) and loss tangent (tan δ) as a function of frequency. Two replicates of each measurement were made.

Emulsifying properties

The emulsifying properties of the rice dough, in the presence and absence of TG and protein isolates, were determined by the method of Pearce and Kinsella (1978). To prepare the emulsion, 2 mL refined sunflower oil and 6 mL freeze-dried samples suspension (0.5%) in 0.1M phosphate buffer (pH 7.0) were shaken together and homogenized in a T18 Ultra Turrax (Wilmington, NC, USA) at 22,000 rpm for 1 min at 20 ºC (Gujral & Rosell, 2004c). Fifty microliter of the emulsion were taken each 10 min and added to 5 mL of sodium dodecylsulphate solution (0.1%, w/v). The absorbance of the diluted solutions was read at 500 nm. The emulsifying activity was expressed as the absorbance measured at 0 min, and the emulsion stability was expressed as ES(%) = (Abs_{60min}/Abs_{0min})x100 (Ahn, Kim & Ng, 2005; Babiker, 2000). Four replicates of each measurement were made.

Quantification of free amino groups

The amount of free amino groups was quantified in the freeze-dried samples in order to confirm the formation of covalent bonds catalysed by TG. This method is based on the reaction between primary amino groups and o-phthaldialdehyde (OPA) (Nielsen, Petersen & Dambmann, 2001; Gujral & Rosell, 2004a). Freeze-dried rice dough (0.1 g) were suspended in 1.0 mL KCl (0.1 M, pH 1.0), vortexed for 10 min and centrifuged for 5 min at 16,000 x g. To 50 μL of the clear supernatant, 250 μL of OPA reagent were added. It was allowed to react for two minutes and the absorbance was determined at 340 nm in a microplate reader. To avoid the ammonia interference, the absorbance at 340 nm was also determined after the precipitation of the proteins with cold trichloroacetic acid (TCA) 20% (w/v). Four hundred fifty microliter of the supernatant were mixed with 50 μL of TCA, kept at 4 ºC for 1h, and centrifuged 10 min at 16,000 x g. Then, to 50 μL of the clear supernatant, 250 μL of OPA reagent were added and, after 2 min, the absorbance was read (Bonet, Caballero, Gómez & Rosell, 2005). The results were calculated against a serine standard curve. Four replicates of each measurement were made.

Statistical analysis

Multiple analysis of variance for the identification of all single effects and interactions was performed by using Statgraphics Plus V 7.1 (Statistical Graphics Corporation, UK). Fisher's least significant differences (LSD) test was used to describe means with 95% confidence.

RESULTS AND DISCUSSION

Effect of different protein isolates on the rice dough viscosity profile during a heating and cooling cycle

Viscosity of rice dough was measured during a heating-cooling cycle to evaluate the effect of the protein isolates from several sources (Fig. 1) and the TG treatment on the pasting properties of the rice flour. Data from the RVA parameters were submitted to the analysis of variance to determine the single effects of the different protein isolates and the transglutaminase (Table 2). The presence of whey protein significantly decreased the peak viscosity of the rice-protein blends by 27% when compared with rice sample. A decrease in the viscosity was also observed when prolamin proteins were added to rice flour (Baxter, Blanchard & Zhao, 2004), likely due to the dilution effect on the starch concentration, since a negative correlation had been established between the protein content and the peak viscosity in rice flour (Lim, Lee, Shin & Lim, 1999; Tan & Corke, 2002). However, the dilution effect could not be

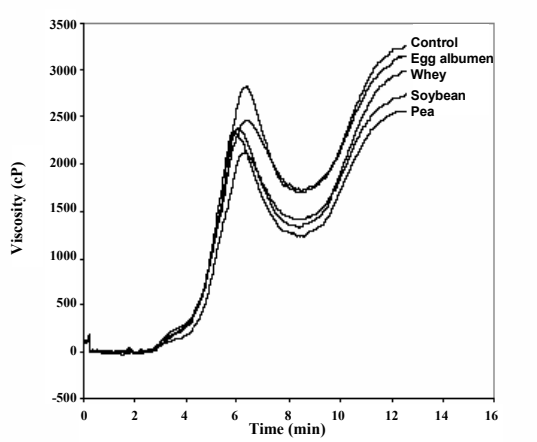

Fig. 1. Effect of different protein isolates on the plots of viscosimetric profile recorded with the rapid viscoanalyzer.

the unique responsible for explaining the decrease in the peak viscosity promoted by whey proteins, because the extent of that was higher than the one observed with the other protein isolates, thus some other properties like the nature of the whey proteins might be responsible of the observed effect. Numerous studies have been reported pertaining to the thermal induced gelation process of the proteins after the aggregation of the polymer chains that induces huge modifications in the rheological behaviour of the proteins, being the gelation process temperature sensitive for each protein (Ngarize, Adams & Howell, 2004; Paulsson, Dejmek & van Vliet, 1990; Puyol, Cotter & Mulvihill, 1999; Ziegler & Foegeding, 1990).

Table 2. Significant single effects of the protein isolates (5 %, w/w, flour-protein blend basis) and the transglutaminase (1 %, w/w, flour-protein blend basis) on the RVA parameters.

RVA parameters	Overall	protein						TG	
	mean	0	1	2	3	4		0	1
Peak viscosity (cP)	2471	2833^{b}	2468^{ab}	2601^{b}	2391^{ab}	2061^{a}	*	2426	2515
Breakdown (cP)	959	1075^{b}	1170^{b}	1147^{b}	714^{a}	689^{a}	*	947	971
Final viscosity (cP)	2988	3338^{c}	2667^{a}	2878^{ab}	3119^{bc}	2940^{ab}	*	2933	3043
Setback (cP)	517	505^{bc}	199^{a}	277^{ab}	728^{cd}	879^{d}	**	507	528

Values followed by different letters in the same row are significantly different (P<0.05).
Protein levels: 0 (without protein isolate), 1 (pea), 2 (soybean), 3 (egg albumen), 4 (whey protein).
TG levels: 0 (without TG), 1 (1% TG).
* p<0.05.
** p<0.01.

The breakdown, related to the ability of the starches to withstand heating at high temperature and shear stress, showed a significant (p<0.05) modification in the presence of different protein isolates, whereas the addition of TG did not induce a significant change in this parameter. The presence of the egg albumen and whey

protein isolates significantly decreased the breakdown by 34 and 36%, respectively, compared to the control in absence of these proteins. Final viscosity was also significantly (p<0.05) modified due to the presence of proteins. Pea, soybean and whey proteins promoted a significant decrease of the final viscosity by 20, 14 and 12%, respectively, when compared to the values of rice flour. However, in this case, the decrease in the viscosity induced by whey protein was lower than the one observed in the peak viscosity. It may be due to the gelation process. Whey proteins do not aggregate easily at room temperature, but they have a high ability to form gel during heating, getting the higher viscosity during cooling. Egg albumen proteins have also this ability, showing higher viscosity than whey proteins (Ngarize et al., 2004). The increase of viscosity during cooling or setback, usually related to the crystallization of the amylose chains when starches are studied, could be affected by the reorganization of the denatured proteins from the protein isolates. In fact, the protein isolates factor resulted in a significant (p<0.01) changes of the setback. Pea and whey proteins significantly modified the setback; but while pea decreased this parameter by 61%, the presence of whey proteins increased it by 74% compared with the values obtained in the absence of protein isolates.

The transglutaminase factor did not result in any significant difference in the RVA parameters of the rice flour-protein isolate blends. Collar and Bollaín (2004) reported a progressive decrease in the peak viscosity and final viscosity in wheat dough with the increase of the TG level up to 0.5%. The different nature of the cereal proteins is greatly responsible for that different behaviour.

Effect of protein isolates and transglutaminase on the viscoelastic properties of rice flour

The viscoelastic properties of the rice dough containing different protein isolates were studied by dynamic oscillatory test. The mechanical spectra of all the samples

showed storage or elastic modulus (G´) values higher than loss or viscous modulus (G´´) at all the frequency range tested, which suggest a viscoelastic solid behaviour of the doughs (Fig. 2). G´ was independent of the frequency in control sample (without protein isolate) and in samples with pea or soybean proteins. Egg albumen and whey protein showed a slight increase in G´ with frequency. G´´ showed lower values at intermediate frequency in control sample and in the presence of whey protein, whereas G´´ of the egg albumen sample showed a slight increase with frequency; in the case of pea and soybean, G´´ was independent of frequency. TG hardly modified the shape of the mechanical spectra (data not shown).

Data from the viscoelastic test were submitted to the analysis of variance to determine the main effects of the protein isolates and the transglutaminase and their interaction (Table 3). The presence of protein isolates significantly (p<0.001) changed the viscoelastic properties of the rice dough, whereas the presence of TG only induced a significant (p<0.001) change of the elastic modulus. A significant (p<0.001) synergistic effect of the protein and TG was also observed on the elastic modulus. The extent of the effect of the protein isolates was greatly dependent on the nature of the proteins. Pea and soybean significantly increased G´ by 39 and 69%, respectively. Whereas the opposite trend was observed with the presence of egg albumen or whey protein, they significantly decreased the value of G´ by 94% in the case of egg albumen and 98% in the case of whey protein. Regarding to viscous modulus, the same tendency than the one observed in the elastic modulus was obtained. Pea and soybean proteins induced a significant large increase of G´´, whereas the opposite trend was observed with the presence of egg albumen or whey protein. The loss tangent was also significantly modified by the presence of egg albumen and whey proteins. This result agrees with the findings of Dogan, Sahin and Sumnu (2005a), who observed higher viscosity in corn-wheat batters when adding soybean protein than in the presence of whey protein or egg albumen. The increase

in the viscosity in the presence of soybean proteins was attributed to its higher water binding capacity (Dogan, Sahin & Sumnu, 2005b).

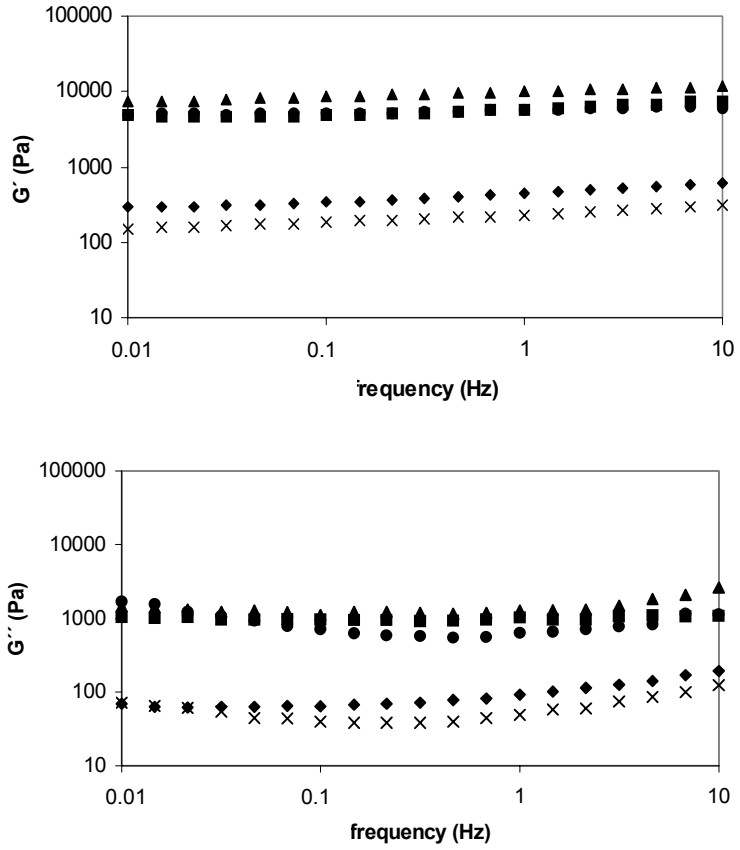

Fig. 2. Variation of elastic (G´) and viscous (G´´) moduli with the frequency for blended rice dough with or without protein isolates. Legends: ● : control; ■ : pea; ▲: soybean; ♦ : egg albumen; **x :** whey proteins.

The addition of TG only induced a significant increase on the elastic modulus of the rice-proteins blends. The other parameters did not show any significant difference. Contradictory results have been reported pertaining to the effect of TG on the viscoelastic properties of different matrix. Larré, Deshayes, Lefebvre and Popineau

(1998) and Larré et al., (2000) and Gujral and Rosell (2004a) reported an increase in G´ values when cereal proteins were treated with TG. An increase in the resistance of the wheat-soybean blends dough was observed when they were treated with TG (Basman, Köksel & Ng, 2003). Köksel, Sivri, Ng and Steffe (2001) reported an increase in the complex modulus (G*) determined by dynamic rheological measurements when wheat doughs containing 10% of bug-damage flour were treated

Table 3. Significant single effects and binary interaction of the protein isolates and the transglutaminase on the viscoelastic parameters.

	G´		G´´		Tan δ	
	F-ratio	P-Value	F-ratio	P-Value	F-ratio	P-Value
Protein isolate	159.26	0.0000	115.90	0.0000	25.02	0.0000
TG	28.70	0.0001	4.24	0.0601	1.04	0.3254
Interaction protein-TG	11.69	0.0003	2.05	0.1460	4.60	0.0156

		Viscoelastic parameters		
	Level	G´ (Pa)	G´´ (Pa)	Tan δ
Overall mean		5372	663	0.176
Protein	0	6460^{b}	719^{b}	0.112^{a}
	1	8992^{c}	1211^{c}	0.145^{a}
	2	10902^{d}	1273^{c}	0.118^{a}
	3	382^{a}	77^{a}	0.205^{b}
	4	126^{a}	33^{a}	0.298^{c}
TG	0	4412^{a}	611^{a}	0.168^{a}
	1	6333^{b}	714^{a}	0.183^{a}

Values followed by different letters in the same column within each factor are significantly different (p<0.05).
Protein levels: 0 (without protein isolate), 1 (pea), 2 (soybean), 3 (egg albumen), 4 (whey protein).
TG levels: 0 (without TG), 1 (1% TG).

with 1.5% TG, but the addition of soy protein isolate did not increase the extent of crosslinking catalysed by TG. Wilcox and Swaisgood (2002) did not observe any crosslinking when whey protein treated with TG were analysed by electrophoresis, but in the presence of reducing agent whey gels adopted more solid-like characteristics. Likely, increasing the accessibility of the amino acid residues in the presence of the reducing agent favours the formation of covalent links within the protein chains. Truong, Clare, Catignani and Swaisgood (2004) did not observe difference in G´ and G´´ values after treating whey proteins with 0.12 unit/g of immobilized TG, but a decrease of G´ was observed when the TG concentration was increased, probably viscoelastic changes can be only observed after an extensive crosslinking of the protein.

Emulsifying properties

The effect of the addition of protein isolates and TG treatment on the emulsifying properties of the proteins was also determined. The presence of egg albumen or whey proteins increased the emulsifying activity of the rice flour, while pea and soybean proteins hardly modified this parameter (Table 4). The stability of the emulsion significantly (p<0.05) decreased when egg albumen and whey proteins were present. Whey proteins are widely used because their emulsifying properties (Hsieh, Regenstein & Rao, 1993), so the presence of these proteins greatly contributes to the emulsifying activity of the rice proteins. Contradictory results have been reported about the emulsion properties of the proteins tested. Jackman and Yada (1989) observed better emulsifying activity and stability of the emulsion with whey protein compared with pea protein. Pearce and Kinsella (1978) also reported higher emulsion activity of the whey protein compared with soybean protein, but egg albumen showed lower emulsion activity than soybean. However, Webb, Naeem and Schmidt (2002) observed better emulsifying properties in soybean than in dairy

proteins. Pea proteins have relatively good emulsifying properties with low emulsion stability compared to the behaviour of the soybean proteins (Tömösközi, Lásztity, Haraszi & Baticz, 2001). These differences may be due to the process followed when producing the protein isolates, since this process can affect the solubility and the

Table 4. Effect of different protein isolates or/and the presence of transglutaminase on the emulsifying properties of the rice flour (none) and the rice flour-protein blends.

Protein	TG (%)	EA	Stability
None	0	0.216 ^{a}	98.1 ^{e}
	1	0.336 ^{b}	35.5 ^{ab}
Pea	0	0.189 ^{a}	94.5 ^{e}
	1	0.320 ^{b}	55.2 ^{cd}
Soybean	0	0.204 ^{a}	83.8 ^{e}
	1	0.347 ^{b}	63.8 ^{d}
Egg albumen	0	0.404 ^{c}	30.8 ^{ab}
	1	0.329 ^{b}	41.6 ^{bc}
Whey	0	0.905 ^{e}	18.8 ^{a}
	1	0.824 ^{d}	26.2 ^{ab}

EA: emulsifying activity, Stability, of the emulsion after 60 min.
Values are the mean of four replicates.
Values followed by different letters in the same column are significantly different (p<0.05).
None: rice flour, pea: rice-pea blend, soybean: rice-soybean blend, egg albumen: rice-egg albumen blend, whey: rice-whey blend.

degree of hydrophobicity, modifying the functional properties of the proteins (Petruccelli & Añón, 1994). In the present study, likely the initial properties of the protein isolates are masked by the dilution effect of the rice flour.

After TG treatment, control sample showed an increase in the emulsifying activity. An increase in this parameter was also showed when the samples with soybean or pea proteins were treated with TG. However, the emulsion stability decreased in these samples. Conversely, the samples with egg albumen or whey protein showed a

decrease in the emulsifying activity but did not modify the emulsion stability after TG treatment. Different behaviours on the emulsifying properties have been reported when proteins are treated with TG. Siu, Ma, Mock and Mine (2002) reported a decrease in the emulsifying activity index and the emulsion stability index when oat globulin was treated with TG. In addition, Ahn et al. (2005) observed a slight decrease in the emulsifying activity but an increase in the emulsion stability after the treatment of cereals flours with TG. The decrease in the emulsifying activity and the emulsion stability caused by TG may be due to the loss of solubility of the crosslinked proteins. The increase in the molecular weight of polypeptide chains may lead to some loss of flexibility and reduces the protein ability to unfold at the oil-water interface (Siu et al., 2002).

Quantification of free amino groups

In order to evaluate the extent of the effect of TG, the free amino groups of the proteins from both rice flour dough and rice-protein blends were quantified.

Transglutaminase (TG) catalyses the reaction between an ε-amino group on protein-bound lysine residues and a γ-carboxyamide group on protein-bound glutamine residues leading to covalent crosslinking of the proteins. Because of the involvement of the amino groups in the crosslinking reaction, a decrease in the amount of these groups would show that TG is catalysing this reaction. A decrease in the amount of the free amino groups was reported by Gujral and Rosell (2004a) and Bonet et al. (2005) when cereal proteins were treated with TG.

As was expected the presence of protein isolates resulted in an increase in the amount of the free amino groups, as a consequence of the increase in the protein content (Fig. 3). After TG treatment, no statistically significant difference was observed in the control (without protein isolates) neither in the presence of pea proteins. However, in the presence of soybean or whey proteins, a decrease in the

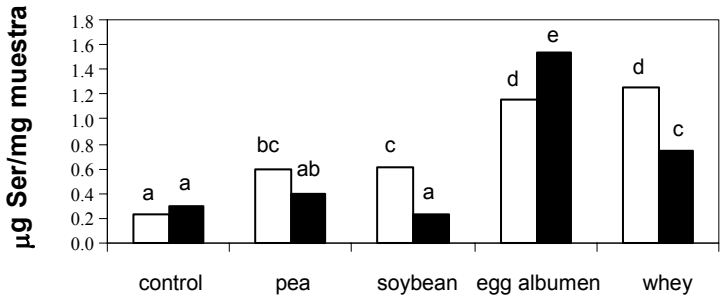

Fig. 3. Effect of the addition of 5 % (w/w, flour-protein blend basis) protein isolate and 1 % (w/w, flour-protein blend basis) transglutaminase on the amount of free amino groups of rice dough. Solid bars: in the presence of TG. White bars: in the absence of TG. Different letters denote statistically significant differences (p<0.05).

amount of free amino groups was observed after TG treatment. The reduction in the amount of amino groups confirmed the protein crosslinking catalysed by TG. This crosslinking may be between homologous or heterologous protein chains. Surprisingly, the sample containing egg albumen protein showed an increase in free amino groups after TG treatment. The increase in the amount of the amino groups might indicate an increase in the solubility of the proteins resulted from the deamidation reaction (Babiker, 2000).

Overall results indicate that the presence of different protein isolates modifies in different extent the properties of the rice flour. The use of transglutaminase changes the viscoelastic behaviour of the rice flour-protein blends getting different trends depending on the protein origin. Therefore, the use of protein isolates and TG broads the applications of rice flour and leads to an increase in the protein content with the subsequent nutritional improvement.

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Capítulo 2

Effect of transglutaminase on the protein pattern of rice, soybean and their blends.

Effect of Transglutaminase on the Protein Pattern of Rice, Soybean and their Blends

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ABSTRACT

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The interactions taking place in composite dough containing rice flour and soybean proteins (5%, w/w) in the presence of transglutaminase, an enzyme with cross-linking activity, were studied by using different electrophoretic analysis. The interaction between rice proteins and soybean proteins was intensified by the formation of new intermolecular covalent bonds catalysed by transglutaminase and the indirect formation of disulfide bonds among proteins. The main protein fractions involved in those interactions were both β-conglycinin and glycinin of soybean and the glutelins of the rice flour, although albumins and globulins were also cross-linked. The addition of soybean proteins to rice flour improves the amino acid balance and also they might play an important role on the rice dough properties because soybean proteins interact with rice proteins yielding protein aggregates of high molecular weight.

Key words: rice, proteins, soybean, transglutaminase, electrophoresis.

INTRODUCTION

Rice has properties that make it an appropriate cereal to use in gluten free cereal products (Gujral et al 2003a, 2003b). The protein content of rice is lower than any other cereals. Rice flour has a protein content around 7.0-8.5%, compared to the 10-15% found in wheat flour. In addition, from the nutritive point of view, rice, as the rest of the cereals, is deficient in lysine, an essential amino acid for the human diet. The combination of cereal proteins with legume proteins has been used for increasing their nutritive value (Ribotta et al 2005). Rice proteins are deficient in lysine but high in methionine, while legume proteins are high in lysine and deficient in methionine, thus they are complementary regarding the essential amino acids (Wolf 1970). The rice-soybean combination would result in gluten free products with better amino acid balance.

The use of rice to obtain fermented products has been very limited because rice proteins are unable to develop gluten, mainly due to the nature of its proteins. The major storage proteins in wheat are prolamins or gliadins (40%) and glutenins (46%) (Orth and Bushuk 1972). These proteins are the main compounds of the gluten, which confers the viscoelastic properties necessary for the dough expansion during fermentation. Conversely, the major storage proteins in rice are the glutelins (65-85%) while prolamins are the minor fraction (Huebner et al 1990).

Soybean is used in food technology for supplying desirable functional properties such as emulsification, fat absorption, moisture holding capacity, thickening, and foaming (Wolf 1970). However, Ribotta et al (2005) reported that soy compounds interfere with gluten formation, decrease dough strength, and diminish dough gas retention capacity, in consequence decreases the bread quality. The negative effects of the soybean might be related to the type of interaction between soy and gluten proteins (Bonet et al 2006, Marco and Rosell, 2008), although it can be improved by a

physical modification of soy flour (Maforimbo et al 2006). In the case of gluten-free bread, the addition of active soybean flour can improve the bread volume, either due to the role of its proteins or the enzyme activities present in the soybean flour (Ribotta et al 2004). The main storage proteins in soybean are globulins, which show two major fractions: 7S or β-conglycinin and 11S or glycinin. Despite the gene of soybean glycinin is derived from common ancestor gene of rice glutelins (Utsumi 1992), rice proteins lack of the functional properties of the soybean proteins. This behaviour is assigned to the way rice glutelins polymerise through disulfide bonds and hydrophobic interactions yielding very large macromolecular structures (Utsumi 1992). Rice and soybean proteins lack of good baking properties, but as they probably have a complementary amino acids profile, some interactions between them could led to a better network.

Transglutaminase (TG) is an enzyme that catalyses the reaction between an ε-amino group on protein-bound lysine residues and a γ-carboxyamide group on protein-bound glutamine residues, leading to the covalent crosslinking of the proteins. This is the predominant reaction, but TG also catalyses two other reactions: in the presence of primary amines, TG crosslinks the amine to a γ-carboxyamide group on protein-bound glutamine residues. In the absence of amine substrates, TG catalyses the hydrolysis of the γ-carboxyamide group of glutamine, resulting in deamidation. The transglutaminase has been used for improving the baking quality of the weak and/or insect-damaged wheat flours (Bonet et al 2005; Caballero et al 2005), bringing about an improvement in the texture of the loaves. In addition, some improvement in the rice protein functionality was observed when they were crosslinked with TG (Gujral and Rosell 2004, Marco and Rosell 2008). Therefore, the use of TG might cross-link rice and soybean proteins to develop a structure similar to the network of gluten. The aim of this study was to understand the interaction between rice and soybean proteins and the cross-linking effect of TG on the rice-soybean blends by

quantifying the proteins content and separating them by using electrophoresis under different conditions.

MATERIALS AND METHODS

Commercial rice flour from Harinera Belenguer SA (Valencia, Spain) was used in this study. The rice flour had moisture, protein, lipid and ash contents of 13.4, 6.5, 0.7 and 0.5%, respectively. The moisture, protein, lipid and ash contents were determined following the Approved Methods 44-19, 46-13, 30-25 and 08-01, respectively (AACC International 2000). Soybean protein isolate was from Trades SA (Barcelona, Spain). The protein isolate had moisture, protein, lipid, ash and carbohydrates (calculated by difference) contents of 6.9, 80.8, 0.2, 3.6 and 8.5%, respectively. Food grade microbial TG (Activa TG) (100 units/g) was provided by Apliena, S.A. (Terrasa, Barcelona, Spain). All reagents were analytical grade.

Rice dough preparation

The dough was made in a 50-g bowl Farinograph (Brabender, Germany), mixing 50 g of rice flour with 90% water (flour basis, corrected to 14% moisture basis) for 15 min. When soybean protein isolate was present, rice flour was replaced by 5% (w/w, flour-protein blend basis) protein isolate. The effect of TG was studied by adding 1% (w/w, flour-protein blend basis) TG. The resulting dough was used for the determination of the protein content and the rest of the dough was frozen and freeze-dried.

Protein quantification

The protein fractions from the doughs were extracted following a sequential extraction with different solvents. Albumin-globulin extraction was conduced by

adding 100 mL of 5% (w/v) NaCl to 20 g of dough. The suspension was homogenized for five minutes and then centrifuged at 5,500 x g for 10 min. After albumin-globulin extraction, the alcohol soluble fraction was extracted from the residue adding 100 mL of 50% (v/v) 1-propanol, following the same procedure as described for the albumin-globulins. The insoluble proteins were extracted with 100 mL of 0.1N NaOH containing 0.5% (w/v) sodium dodecyl sulfate (SDS) and 0.6% (v/v) β-mercaptoethanol (ME) (Sugimoto et al 1986; Ju et al 2001). Each extraction was repeated twice to increase the protein extraction. The supernatants were collected. Protein contents in the doughs and protein fractions were determined following the micro-Kjeldahl method (Approved Method 46-13, AACC International 2000), using 5.95 as the protein conversion factor.

SDS-PAGE protein electrophoresis

Electrophoresis was conducted under nonreducing and reducing conditions to determine differences due to the presence of disulfide bonds. For total protein extraction under non reducing conditions, a buffer containing 0.063M tris(hydroxymethyl) aminomethane (Tris/HCl, pH 6.8), 2% (w/v) SDS, 10% (v/v) glycerol and 0.01% (w/v) bromophenol blue was added to freeze-dried dough (1 mL of buffer/50 mg of rice dough and 1 mL/30 mg of rice-soybean blend) and soybean protein isolate (1 mL/5 mg). Suspensions were vortexed for 2.5 hr, heated in a boiling-water bath for 5 min, and cooled at room temperature. Then they were centrifuged at 400 x g, for 5 min. The proteins remained in the supernatants. Sequential extraction of the different protein fractions was made using the solvents previously described. Under reducing conditions, the procedure was the same as the non reducing conditions, but the buffer solution also contained 3% (v/v) β-mercaptoethanol.

Supernatants extracted under non reducing conditions were used for simple SDS-PAGE and multistacking SDS-PAGE (preparative and analytical). The supernatants extracted under reducing conditions were used only for analytical SDS-PAGE.

SDS-PAGE was performed in 12% separating gels with 4% stacking gels according to Laemmli (1970). In multistacking electrophoresis (Khan and Huckle 1992), two stacking gels of 4 and 8% (w/v) acrylamide concentration (0.108 and 0.216% w/v bisacrylamide concentration, respectively) and one resolving gel of 12% (w/v) acrylamide (0.48% w/v bisacrylamide) were prepared. Gels of 0.75-mm width were prepared for analytical purposes, and gels of 1.5-mm width were used for preparative analysis. A Mini Protean II slab cell (Bio-Rad Laboratories, Richmond, CA) vertical unit was used**.** The standard proteins were from Bio-Rad (low range, Bio-Rad Laboratories, Hercules, USA) and consisted of phosphorilase b (97,000), bovine serum albumin (66,000), ovalbumin (45,000), carbonic anhydrase (31,000), soybean trypsin inhibitor (21,500) and lysozyme (14,400). The gels were stained with 0.25% Coomassie Brilliant Blue R in methanol-water-acetic acid (4:5:1, v/v) and were de-stained in the same solvent, excluding the dying reagent. The gels from preparative multistacking were not stained, instead, they were cut initially into three pieces (corresponding to different acrylamide concentration) that were separately submerged into buffer solution containing ME, then they were vortexed for 48 hours at ambient temperature. The resulting mixtures were placed in a water bath at 100 °C for 10 min. Protein composition was analyzed by SDS-PAGE (stacking gel of 4% w/v, acrylamide and resolving gel of 12% w/v, acrylamide). Runs were performed in the same equipment as described above. Gels were quantified using an Image Master VDS (Pharmacia Biotech, USA) equipped with ImageMaster VDS software providing the integrated optical density (IOD) values.

Statistical analysis

Multiple sample comparison was used for the statistical analysis of the results (Statgraphics Plus 5.1, Statistical Graphics Corporation, UK). Fisher's least significant differences (LSD) test was used to describe means with 95% confidence.

RESULTS AND DISCUSSION

Quantification of the protein fractions

To determine the protein fractions affected by the cross-linking activity of the TG, the protein content in each protein fraction was determined by the Kjeldahl method. As it was expected, the glutelins were the major protein fraction in rice, representing about 77.8% of the total proteins (Table 1). The content in salt-soluble proteins (albumin-globulin) was 15.5% and the alcohol-soluble protein (prolamin) content 4.3%. Those values fall within the results reported previously (Gorinstein et al 1999; Ju et al 2001). When 5% (w/w) soybean protein isolate was blended with rice flour, the proportion of salt-soluble proteins increased up to 18.0%, thus soybean proteins were mainly extracted with the albumin-globulin fraction. The amount of alcohol-soluble proteins decreased as a consequence of the rice protein replacement by soybean, whereas SDS-soluble fraction showed barely the same value, indicating that soybean proteins were partially extracted with the SDS solvent overcoming the dilution effect. The major part of the soybean proteins has been classified as globulins (Wolf 1970; Gorinstein et al 1999), the increase observed in the SDS-soluble fraction might be ascribed to the production process of the protein isolate because it may cause physical and chemical changes and, therefore, may affect the solubility of the proteins (Arrese et al 1991).

In the presence of TG, a decrease in the extraction of the albumin/globulin and glutelins and an increase in the residue content were observed. The more affected

fractions were the salt soluble proteins in both samples (rice and rice-soybean blends) and the SDS soluble proteins in the rice-soybean blend. Similar behaviour

Table 1. Effect of transglutaminase (1%, w/w, solid basis) on the protein fraction content of rice flour dough and composite doughs containing rice flour and soybean protein (5%, w/w).

	Salt soluble proteins (%)	Alcohol soluble proteins (%)	SDS soluble proteins (%)	Final residue (%)
Rice	15.5 b	4.3 b	77.8 b	2.4 a
Rice+TG	10.0 a	4.9 b	77.4 b	7.8 b
Rice-soybean blend	18.0 c	3.4 a	77.2 b	1.4 a
Rice-soybean blend+TG	10.4 a	2.9 a	59.1 a	27.7 c

Means within columns followed by the same letter were not significantly different (P<0.05).

was obtained when pea proteins were enzyme cross-linked with rice flour proteins (Marco et al 2007). Likely, the cross-linking action of the TG induced changes in the extractability of the proteins. The extent of the TG effect was higher in the rice-soybean protein blend, where the residue increased from 1.4% in absence of TG to 27.7% after TG treatment. The rice-soybean protein blend was a better substrate for the TG than the rice alone, which could be related to the lysine content that necessary for the cross-linking reaction catalyzed by TG. Rice has lysine values of about 4.0 g/16.8 g of N (Cagampang et al 1976) compared with the soybean lysine values of about 6.0 g/16.8 g of N (El-Moniem et al 2000). In rice the lysine amino acids are mainly concentrated in the albumin fraction (Villareal and Juliano 1978).

SDS-PAGE analysis

SDS-PAGE was performed in different conditions for evaluating the nature of the interactions between proteins from rice and soybean due to the TG activity. The SDS-PAGE analysis was performed under nonreduced and reduced conditions. When

electrophoresis was performed in nonreduced conditions (without ME), two major bands at 53,700 and 22,400 (molecular weight obtained from the densitometric analysis) were found in the rice sample and also some polypeptides of high molecular weight that were unable to enter the stacking and separating gels (Fig. 1A). In the presence of TG, a decrease in the intensity of the protein bands was observed, with the exception of a band at 35,300 that showed a great increase in the intensity (554-1,193 IOD units) after the TG treatment (Fig. 1A). Probably, the formation of a new polymer due to the cross-linking reaction produced this increase in the band at 35,300.

Soybean protein isolate, showed the characteristics bands of this legume: α′, α and β subunits at 85,000, 76,200 and 51,400 corresponding to the β-conglycinin, and A and B subunits at 37,400 and 22,500 corresponding to the glycinin (Fig. 1A) (Ribotta et al 2005, Tang et al 2006). The lane of the rice flour-soybean protein blend (Fig. 1A) did not show the band corresponding to the A glycinin subunit of soybean, probably due to it is prone to interact with other proteins. In the presence of TG, this sample also showed a decrease in the intensity of the bands (Fig 1A). The α′ (MW 86,800) and α (MW 79,000) subunits corresponding to the β-conglycinin did not appeared after TG treatment. The band at 33,700 showed higher intensity than that without TG treatment, likely due to the aggregation of small polypeptides. Soybean has been reported as a good substrate for TG, giving better results than wheat and barley (Basman et al 2002). The cross-linking by TG involves both β-conglycinin and glycinin of soybean, mainly affecting the acidic subunits of the β-conglycinin, because the basic subunits are not readily accessible for TG (Tang et al 2006). In addition, the treatment with TG induced a decrease in the protein located on the top of the stacking and separating gels. Protein extractability can be reduced due to both the protein cross-linking catalyzed by TG or the indirect formation of disulfide bonds, because the

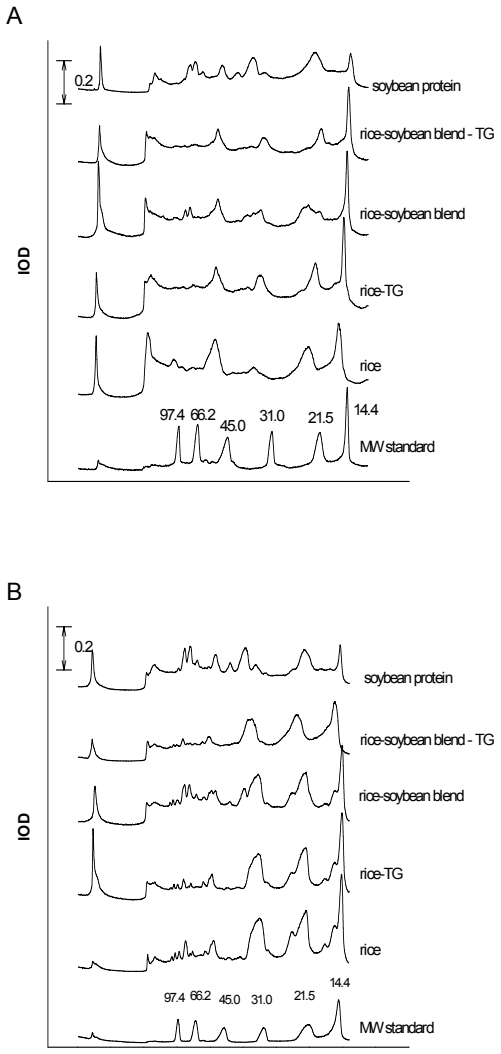

Figure 1. Electrophoregrams obtained from the analysis of the SDS-polyacrylamide gels of the proteins from rice and rice-soybean protein blends in the absence and the presence of 1% (w/w) transglutaminase. The values in the MW standard are expressed in kDa. (A) unreduced conditions; (B) reduced conditions. IOD: Integrated optical density.

crosslinking reaction may draw the sulfur containing amino acids, making it easier to form these bonds (Gujral and Rosell 2004; Marco et al 2007).

Under reducing conditions, a higher number of bands corresponding to rice and soybean proteins was observed (Fig 1B). In the rice sample, the intensity of the band at 53,000, which was the major band in nonreducing conditions, showed a great decrease when electrophoresis was performed in the presence of ME, indicating the existence of disulfide bonds in that fraction (Fig 1B). The three major protein bands of the rice appeared at 15,100, 22,300 and 32,700 that are very close to those reported by Steenson and Sathe (1995) in the *Basmati* rice (14,500, 20,400 and 33,100) and by Villareal and Juliano (1978) in *Indica* rice (16,000, 25,000 and 38,000). Regarding the rice-soybean proteins blends (Fig. 1B), it was possible to observe the band corresponding to the A glycinin subunit, which was not observed under nonreducing conditions, confirming the interaction of this subunit by disulfide bonds yielding polymers with higher molecular weight.

In rice and rice-soybean blend samples, the TG activity promoted an increase in the protein unable to enter the stacking and separating gels and also a decrease in the intensity of the bands, with the exception of the band around 32,700 in the rice sample and the bands around 34,700 and 23,400 in rice-soybean blend, where the relative intensity showed an increase in the presence of TG (Fig 1B), again probably due to the aggregation of small polypeptides. The bands more affected by TG were MW about 76,800 and 65,700 (α and α' conglycinin subunits**)**, 51,400 (β conglycinin subunit), and 37,900 (A glycinin subunit) in rice-soybean blends. Those bands were not present after TG treatment, which means these proteins were involved in the cross-linking reaction. Several authors have studied cross-linking between glycinin and conglycinin (Ikura et al 1980) and also with other globular proteins (Yildirim et al 1996), explaining that both soybean proteins were able to form polymers through covalent bonds catalysed by TG and that the crosslinking of glycinin proceeded faster

than that of conglycinin (Ikura et al 1980). In addition, the basic subunits of glycinin remain almost intact after TG treatment, while the acidic subunits are the most affected proteins (Ikura et al 1980; Tang et al 2006). This difference between the reactivity of the acidic and basic subunits from glycinin could be related to the native structure of glycinin, because the basic subunits of glycinin are buried in the interior of hexamers of glycinin, which are difficult to access at the active site of the TG (Nielsen 1985; Tang et al 2006). In addition, compared with the acidic polypeptides, the basic polypeptides contain relatively low levels of glutamine and lysine, the necessary amino acids for TG crosslinking reaction (Nielsen 1985).

The extent of the TG effect in each protein fraction, obtained from a sequential extraction, was determined. TG promoted an increase in the band intensity on the top of stacking and separating gel in the albumin-globulin fraction of the rice sample (Fig. 2A, lane 3). Conversely, the band at 90,800 disappeared after TG treatment and a decrease in the intensity was observed in the bands with lower MW. The trend of the albumins-globulins in the rice-soybean protein blend treated with TG was the same (Fig. 2B, lane 3). The majority of the bands in this fraction were from rice, although a disappearance of a band at 37,000 corresponding to soybean protein was noticed, as was the disappearance of the soybean bands at 66,200-97,400 kDa. This indicates that the TG cross-linking proteins yield an increase in the molecular weight of the polymers retained on the top of the stacking and separating gel. The prolamins fraction showed only a slight decrease in intensity due to the TG activity.

The glutelins extraction was made in two steps: 1) in the absence of ME (non-reducing conditions) and 2) in the presence of ME for increasing the protein extraction. Therefore, besides the cross-linking catalyzed by TG, the activity of the enzyme also increased the disulfide bonds between proteins (Gujral and Rosell 2004; Marco et al 2007) which made it necessary to use a reducing agent to favour its extraction. Reducing and nonreducing conditions of extraction produced the same

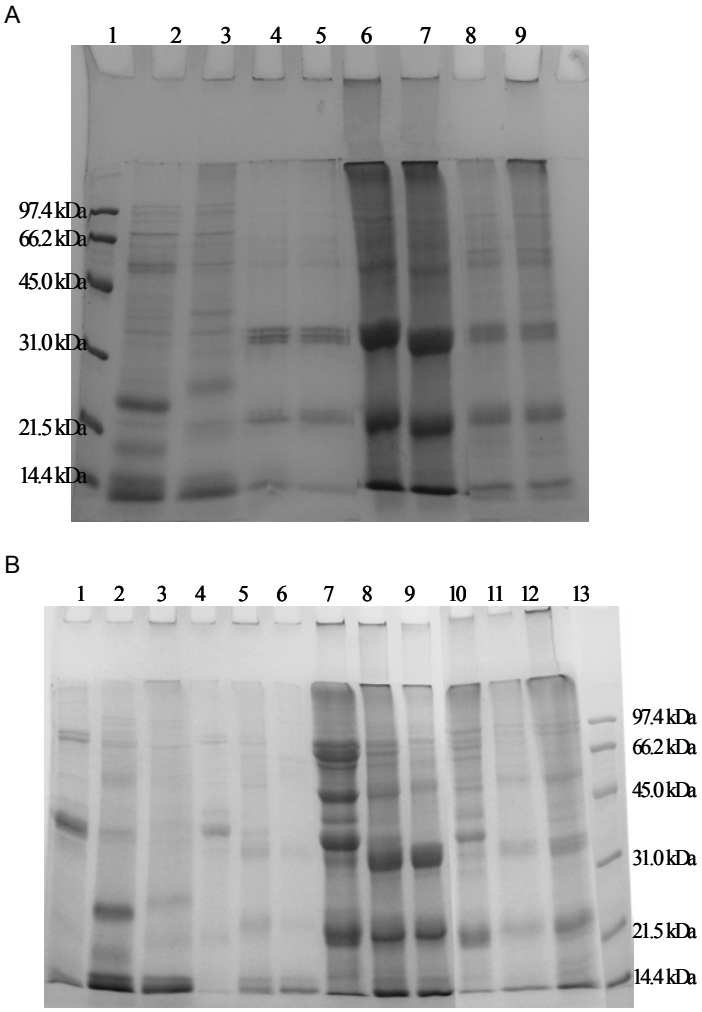

Figure 2A. SDS-PAGE analysis of the protein fractions of rice in the absence (lanes 2, 4, 6, 8) and the presence of TG (lanes 3, 5, 7, 9). MW standard (lane 1), albumins-globulins (lanes 2, 3), prolamins (lanes 4, 5), glutelins step 1 (lanes 6, 7) and glutelins step 2 (lanes 8, 9). **2 B**. SDS-PAGE analysis of the protein fractions of soybean protein isolate (without TG) (lanes 1, 4, 7, 10) and of rice-soybean blend in the absence (lanes 2, 5, 8, 11) and the presence of TG (lanes 3, 6, 9, 12). Salt soluble proteins (lanes 1, 2, 3), alcohol soluble proteins (lanes 4, 5, 6),

SDS soluble proteins step 1 (lanes 7, 8, 9), SDS soluble proteins step 2 (lanes 10, 11, 12). MW standard (lane 13).

electrophoresis pattern. Rice sample showed two major bands with MW of about 34,000 and 22,700 (Fig 2A, lanes 6 and 8) that corresponded to the acidic and basic polypeptides of rice glutelins, respectively (Villareal and Juliano 1978; Steenson and Sathe 1995; Jahan et al 2005).

Soybean protein isolate showed the β-conglycinin and glycinin subunits (Fig. 2B, lanes 7 and 10), although it was expected that these proteins were extracted in the salt- soluble fraction because globulins are the major storage proteins in soybean. Presumably, the change in the solubility of these proteins was due to the process of the soybean protein production, which can modify the characteristics of the resulting protein isolate (Arrese et al 1991). In rice-soybean protein blend (Fig. 2B, lanes 8 and 11), the basic polypeptides of the rice glutelins appeared at the same molecular weight as the basic subunit of the soybean because the genes of both proteins come from a common ancestor gene (Utsumi 1992). In addition, a small amount of high molecular weight components (around 58,000), likely comprising residual albumins and globulins, was observed together with a major band at approximately 14,000. The low molecular weight fraction probably was a prolamin polypeptide, typically reported as the principal contaminant of glutelin preparations from rice (Krishnan and Okita 1986).

In the TG-treated samples of rice-soybean protein blend, the bands with molecular weight about 70,300 and 40,700, both from soybean disappeared after the TG treatment and the intensity of the bands was higher under the reducing conditions, probably because more proteins remained insoluble in the TG sample after the extraction in non reducing conditions (Fig. 2B, lanes 9 and 12). The effect of the TG in the SDS-soluble fraction was more noticeable in the rice-soybean protein blend than in rice sample, where the effect of the enzyme was hardly noticed in the low

molecular weight proteins. In the SDS-soluble fractions, the intensity on the top of the stacking and separating gel was higher in the absence of TG when the extraction was made without ME (Fig. 2B, lane 8). Oppositely, when the reducing agent was used to extract the rest of the SDS-soluble proteins, more aggregates were observed on the top of the gel in the sample with TG (Fig. 2B, lane 12) that indicates the formation of disulfide bonds in great extent in samples with TG. The protein aggregates that remained on the top of the stacking and separating gel in the TG-treated samples confirms the intermolecular crosslinking between the proteins extracted with SDS.

The cross-linking reaction among proteins catalysed by TG may be intermolecular or intramolecular, only resulting in higher molecular weight of the proteins when intermolecular linkages are formed (Basman et al 2002). Numerous studies related to the effect of TG reported a decrease in the intensity or disappearance of some bands of the proteins and an increase in the protein aggregates retained on the top of the stacking and separating gel, supporting the intermolecular crosslinking (Yildirim et al 1996; Babiker 2000; Basman et al 2002; Mujoo and Ng 2003; Fan et al 2005; Marco et al 2007).

The increase in the molecular weight of the proteins and, therefore, the increase in the interactions between proteins (disulfide bonds or other covalent interactions) modifies or improves the viscoelastic and functional properties of rice-soybean doughs (Marco and Rosell 2008). A relationship has been also established between the increase in the proportion of HMW proteins of soybean proteins and their potential for being used in wheat flour breadmaking (Maforimbo et al 2006).

Multistacking SDS-PAGE

The proportion of proteins (relative integrated optical density [IOD]) retained in the multistacking SDS-PAGE as a function of molecular weight is shown in Table 2. In the absence of TG, the blend of soybean with the rice flour induced an increase in the

proportion of proteins with HMW (retained in the 4 and 8% gels) was observed, probably due to the association of the soybean proteins with rice proteins, yielding protein aggregates. In the presence of TG, a decrease in the proportion of aggregates with higher MW was observed in the presence of TG, as a consequence of a

Table 2. Relative IOD (integrated optical density) of the protein retained in the different concentrations of the multistacking gel. Rice-soybean blends were prepared by replacing 5% (w/w) of rice flour with soybean protein. In the transglutaminase treated samples, 1% (w/w) of transglutaminase was added.

	Relative IOD		
	4%	**8%**	**12%**
Rice	8.1 b	8.0 b	84.0 c
Rice+TG	2.8 a	2.3 a	94.9 d
Rice-soybean blend	17.3 d	13.1 d	69.6 a
Rice-soybean blend +TG	14.0 c	10.1 c	75.8 b

Means within columns followed by the same letter were not significantly different (P<0.05).

decrease in the solubility of the aggregates crosslinked by the enzyme. Only the monomers with lower MW could be extracted and separated in the 12% gel.

The preparative multistacking allows determining the proteins retained in the different gel concentrations (4, 8 and 12%). In rice and rice-soybean protein blends, the same protein bands were observed in the three concentrations of the gels, with the exception of two bands (*) that only appeared in the lane corresponding to the 12% gel (Fig. 3A and B). These proteins were from rice and they did not contribute to the formation of HMW aggregates in the rice-soybean protein blend. However, in the presence of TG, the intensity of these bands decreased (Fig. 3A and B, lane 6), likely due to their cross-linking forming insoluble aggregates. That result was supported by the increase in the intensity on the top of the stacking and separating gels in the lane

corresponding to 4% gel (Fig. 3A and B, lane 4). Therefore, the intensity of all bands decreased in the presence of TG, and some of the bands disappeared due to the crosslinking, being the HMW proteins most affected.

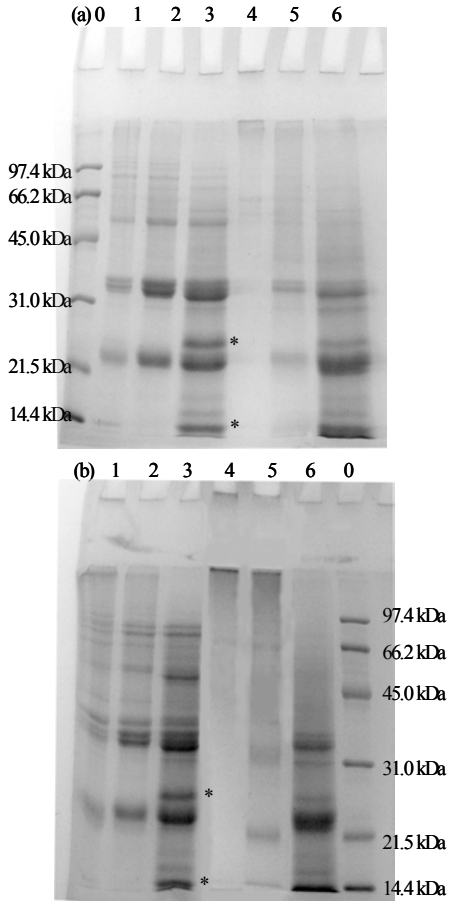

Figure 3. Preparative multistacking SDS-PAGE of rice (a) and rice-soybean blend (b). Acrylamide/bisacrylamide concentrations of 4, 8 and 12% without TG (lanes 1, 2 and 3, respectively) and with TG (lanes 4, 5 and 6) and MW standard (lane 0).

CONCLUSIONS

The addition of soybean proteins to rice flour provides a protein-enriched dough with better amino acid balance and those proteins also might play an important role on the rice dough properties because soybean proteins interact with rice proteins, yielding HMW protein aggregates. The interaction between rice proteins and soybean proteins can be intensified by the formation of new intermolecular covalent bonds catalysed by TG and also the indirect formation of disulfide bonds. The main protein fractions involved in those interactions are both β-conglycinin and glycinin of soybean and the glutelins of the rice flour, although albumins and globulins were also crosslinked.

ACKNOWLEDGEMENTS

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Capítulo 3

Effect of microbial transglutaminase on the protein fractions of rice, pea and their blends.

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Effect of microbial transglutaminase on the protein fractions of rice, pea and their blends

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Abstract

BACKGROUND: Transglutaminase (TG) is a transferase that has been used for crosslinking proteins. In general, those interactions are promoted within proteins of the same nature, and very few studies have been conducted for creating new bonds between proteins from different sources catalized by TG. The effect of transglutaminase on the protein fractions of rice flour, pea protein isolate and their blends was studied by using different electrophoretic analysis (simple sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and multistaking SDS-PAGE under reducing and non-reducing conditions).

RESULTS: TG induced the disappearance of numerous protein bands as a consequence of the formation of large protein polymers, linked by isopeptidic and disulphide bonds, with reduced solubility. The main protein fractions involved in those interactions were the albumins and globulins, from the pea protein isolate, and the rice flour; and the glutelins were also crosslinked.

CONCLUSION: Composite flours containing the rice flour and the pea protein isolate are proposed for obtaining a protein-enriched dough with better amino acid balance. Also a protein network formed of proteins aggregates of high molecular weight can be created in the presence of transglutaminase.

Keywords: rice; proteins; pea; transglutaminase; electrophoresis

INTRODUCTION

Among gluten-free cereals, rice is the most appropriate for producing fermented products because of its unique properties.1-3. Rice flour has a soft taste, is colourless, and has low levels of sodium, easily digestible carbohydrates and low hypoallergenic properties. Nevertheless, despite the described advantages, rice flour shows an important drawback from the technological point of view, since its proteins do not develop the appropriate viscoelastic network necessary to retain the gas produced during the fermentation process, resulting in low-quality products.

The addition of transglutaminase (TG) to rice dough has been reported as an alternative to improve the quality of fermented rice-based bread by creating a protein network.[4] The use of this enzyme allows a decrease in the quantity of structuring agents needed to obtain rice bread of acceptable quality. TG (protein-glutamine γ-glutamyltransferase, EC 2.3.2.13) is an enzyme that catalyses the reaction between an ε-amino group on protein-bound lysine residues and a γ-carboxyamide group on protein-bound glutamine residues, leading to covalent crosslinking of the proteins. This crosslinking may be inter- or intramolecular, yielding an increase in the molecular weight of the protein molecules when intermolecular bonds are formed. [5,6] This reaction has been used for creating crosslinks among proteins from different sources. Proteins such as wheat gluten, soybean proteins, whey proteins, myosin and actomyosin have been reported to be acceptable substrates for TG, [7-10] modifying the properties of the proteins and thus broadening their applications in foods.

Gluten free products are frequently produced with the addition of various proteins to increase their nutritional value. [11-13] Legumes are a good supplement for cereal based foods because they increase the protein content, complementing the nutritional value of the cereal proteins. Cereals are deficient in lysine, one of the essential amino acid for the human diet, while legumes show a high content of this amino acid. Simultaneously, cereal proteins are able to complement legume proteins in the

essential amino acid methionine.[14] Although the most used legume protein is the soybean protein because of its valuable functional properties, pea proteins can also be successfully used in bakery products, obtaining an enrichment in proteins and improving the biological value.[15] Moreover, from the technological point of view the addition of proteins from different sources to rice flour increases the possibilities for crosslinking by TG, since the addition of proteins with a high content of lysine groups increases the reactivity of the proteins. It has been reported that proteins from different sources interact with wheat proteins in the presence of TG, changing the rheological properties of the wheat flour dough and also their microstructure.[10] The creation of new crosslinks and in consequence the formation of protein polymers might extend the use of rice flour. It has been reported that the addition of pea protein isolate and TG to rice flour produces an increase in storage and loss moduli of doughs.[16] Protein crosslinking catalysed by TG yielded doughs with more elastic and viscous behaviour. However, changes in the protein fractions of rice flour and pea proteins as affected by crosslinking with TG have not been well studied.

The goal of this study was to determine the effect of TG on the protein fractions of rice flour and pea proteins by quantifying these fractions, and to understand the nature of the interaction between proteins in the rice-pea protein blends after TG treatment using sodium dodecyl sulfate-polyacrilamide gel electrophoresis (SDS-PAGE) under different conditions.

MATERIALS AND METHODS

The commercial rice flour used was from Harinera Belenguer SA (Valencia, Spain). The rice flour had moisture, protein, lipid and ash contents of 134 g kg^{-1}, 75 g kg^{-1}, 9 g kg^{-1} and 6 g kg^{-1} (dry basis), respectively. Pea protein isolate was from Trades SA (Barcelona, Spain) and had moisture, protein, lipid, and ash contents of 67 g kg^{-1}, 848 g kg^{-1}, 9 g kg^{-1}, and 45 g kg^{-1} (dry basis), respectively. Microbial transglutaminase

from *Streptomyces* spp. from Ajinomoto Co. Inc. (Tokyo, Japan) (100 units g^{-1}) was kindly supplied by Apliena, S.A. (Terrasa, Barcelona, Spain). All reagents in this study were of analytical grade.

Rice dough preparation

Dough was made in a 50g bowl Farinograph (Brabender, Germany). Rice flour (50 g) was mixed with 45 mL water at a controlled temperature (30 °C) for 15 min. Previous studies stated that this mixing time was sufficient for the enzyme reaction to occur.[4, 8-10] In samples containing pea protein isolate, rice flour was replaced by 5% (w/w, flour-protein blend basis) pea protein isolate. TG, when added, was incorporated at a level of 1% (w/w, flour-protein blend basis), which corresponded to 1.0 TG unit/g of flour-protein blend. The doughs obtained were used for determination of protein content and remaining doughs were frozen and freeze-dried.

Protein amount determination

Protein fractions were extracted following a sequential extraction using different solvents, following the method of Ju *et al.,*[17] with slight modifications. Briefly, the albumin-globulin fraction was obtained by suspending 20 g of dough in 100 mL of 5% sodium chloride; it was then homogenized for 5 min and centrifuged at 5500 x *g* for 10 min. The procedure was repeated twice for better extraction and the supernatants were collected. The prolamin fraction was then extracted by adding 100 mL of 50% 1-propanol to the residue, following the same procedure as in the albumin-globulin extraction. Finally, the glutelins were extracted adding 100 mL of 0.1 mol L^{-1} NaOH - containing 0.5% SDS and 0.6% *β*-mercaptoethanol (ME) - to the residue.

The protein content of the supernatants and in the final residue were determined by the micro-Kjeldahl method approved by the AACC.[18] The N:protein conversion factor used was 5.95.[19]

SDS-PAGE protein electrophoresis

Protein extraction

Total proteins were extracted under non-reducing and reducing conditions. The extraction under non reducing conditions was made using the following buffer solution: 0.063 mol L^{-1} tris(hydroxymethyl)aminomethane (Tris-HCl) pH 6.8, 2% (w/v) SDS, 10% glycerol, and 0.01% (w/v) bromophenol blue. One millilitre of the buffer was added to the freeze-dried doughs (50 mg rice or 30 mg in the case of rice-pea protein blend) or to the pea protein isolate (5 mg). The suspensions were then vortexed for 2.5h and heated in a boiling-water bath for 5 min. After cooling at room temperature, samples were centrifuged for 5 min at 4000 x *g*. The method followed for the extraction under reducing conditions was as described for non-reducing conditions, but buffer solution also contained 3% (v/v) ME as reducing agent. The supernatants containing the proteins were used to perform electrophoresis.

Protein fractions were extracted following a sequential extraction with the same solvents as used in the determination of proteins amount previously described.

Electrophoresis analysis

Simple SDS-PAGE was performed using the supernatant obtained under reducing and non-reducing conditions. The supernatants obtained under non-reducing conditions were also used for multistacking SDS-PAGE (analytical and preparative).

SDS-PAGE was performed in 12% resolving gels with 4% stacking gels according to Laemmli.[20] In multistacking electrophoresis, the acrylamide concentrations of the gels were 4% and 8% (w/v) for the stacking gels and 12% (w/v) for resolving gels.

A Mini Protean II Slab Cell (Bio-Rad Laboratories, Richmond, CA, USA) vertical unit was used. Molecular weight standards were from BioRad (low range, BioRad Laboratories, Hercules, CA, USA) and consisted of phosphorylase b (97.4 kDa), bovine serum albumin (66.2 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa),

soybean trypsin inhibitor (21.5 kDa) and lysozyme (14.4 kDa). The gels were stained with 0.25% Coomassie Brilliant Blue R in methanol-water-acetic acid (4:5:1 v/v) and were destained in the same solvent excluding the dying reagent. The gels from preparative multistacking were not stained; instead, they were cut up into pieces and separately submerged into buffer solution containing ME. Then they were vortexed at room temperature for 48 h and the resulting mixtures were placed into a water bath at 100 ºC for 10 min. Protein composition was analyzed by SDS-PAGE (stacking gel of 4% (w/v) acrylamide and resolving gel of 12% (w/v) acrylamide). Runs were performed in the same equipment as described above. Gels were analysed by an Image Master VDS (Pharmacia Biotech, Piscataway, NJ, USA), equipped with Image Master VDS software providing the integrated optical density (IOD) values.

RESULTS AND DISCUSSION

Protein content in the protein fractions

Sequential extraction of the different protein fractions (albumin, globulin, prolamin and glutelin) and their further quantification provided information about the protein fractions from both the pea protein isolate and the rice flour involved in crosslinking catalysed by TG. The major protein fraction in the rice sample was glutelin, representing 77.8% of total protein (Table 1), whereas the prolamins were the minor fraction. The albumin-globulin fraction represented 15.5% of total protein in the rice flour. The proportion of each fraction agrees with the results reported previously.[17,19]

When pea protein isolate was present in the rice-protein blend, rice proteins (0.4 g) were replaced by pea proteins (4.2 g), leading to protein enrichment in the blend. The addition of the pea protein isolate produced an increase in the proteins extracted in the albumin-globulin fraction. Conversely, a decrease in the proportion of protein extracted in the glutelin and prolamin fractions was observed. Nevertheless, taking into account that pea proteins have been classified as globulins,[21] the resulting value

Table 1. Study of the effect of the addition of 1% (w/w) TG on rice and rice-pea blend doughs by the quantification of the protein nitrogen content in the different protein fractions (albumin-globulin, prolamin and glutelin).

	Albumin-Globulin (%)	Prolamin (%)	Glutelin (%)	Final residue (%)
Rice	15.5	4.3	77.8	2.4
Rice+TG	10.0	4.9	77.4	7.8
Rice-pea blend	26.8	3.5	68.1	1.6
Rice-pea blend+TG	11.5	3.0	60.2	25.3

for the glutelin fraction of the rice-pea blend was too high. That result could be attributed to changes in the solubility properties of the proteins, since the extraction process of the protein isolate can affect their properties.[22,23] The proportion of the final residue in the rice-pea protein blend was lower than in the rice sample; thus, the pea proteins were more soluble than the rice proteins under the conditions used.

Activity of TG was evidenced by a change in the protein fraction pattern. A decrease in the albumin-globulin fraction was observed in both samples (rice flour and the rice-pea protein blend) and in the glutelin fraction extracted from the rice-pea protein blend. In the presence of pea protein isolate the effect of TG was greater than in its absence, and the more affected fraction was the albumin-globulin. Pea proteins have a higher lysine content, which might favour the reaction of TG because this amino acid is necessary for the crosslinking reaction catalysed by this enzyme. Likewise, the presence of pea proteins might induce either a different aggregation of the rice proteins or hinder their aggregation, improving the accessibility of the enzyme to the lysine or glutamine groups involved in crosslinking.

The presence of pea induced a greater effect of TG on all the protein fractions, yielding a reduction in the protein extracted, with a simultaneous increase in the protein content of the final residue. A substantial increase of the protein content in the final residue was observed in both samples after TG treatment. This increase was higher in the rice-pea protein blend, where the proportion of protein in the residue increased from 1.6% to 25.3%. Therefore, crosslinking catalysed by TG lead to the formation of large protein polymers with a reduced solubility of the news polymers formed.

SDS-PAGE analysis of the total proteins

Electrophoresis in polyacrylamide gels was performed in order to characterize the protein polymers formed as a consequence of TG treatment. The electrophoresis was performed under different conditions with the purpose of understanding the nature of the interactions among the proteins due to the activity of this enzyme.

Under non-reducing conditions, the major bands observed in the rice sample and rice-pea blend were those of molecular weight 14.5-15.7, 22.4-23.5 and 52.8-53.7 kDa, corresponding to rice proteins (Fig. 1a). In both samples in the presence of transglutaminase, a decrease in the intensity or a disappearance of the protein bands was observed, with the exception of the 35.0 kDa band, whose intensity increased probably due to the formation of new polymers with different solubility, and the 15.0 kDa band, probably as a result of peptides that were unable to aggregate due to the TG activity at various points of the protein chain. The extent of the intensity decrease was greater in the case of rice-pea blends, which agrees with the results previously described for the protein quantification. A decrease in the protein retained on the top of the stacking and resolving gels was also observed in the presence of transglutaminase. The protein polymers unable to enter the gel in the absence of TG

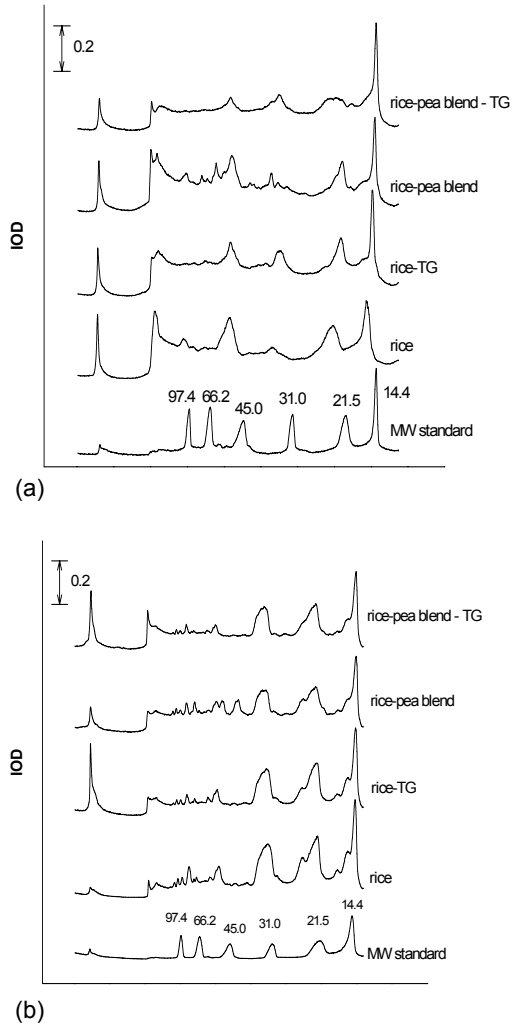

Figure 1. Electrophoregrams obtained from the analysis of SDS-polyacrylamide gels of proteins from rice and rice-pea blends in the absence and the presence of 1% (w/w) TG. The values in the molecular weight standard are expressed in kDa. (a) unreduced conditions; (b) reduced conditions.

increased their MW due to the TG activity, yielding polymers of greater size and lower solubility, which were not extracted and remained in the residue.

In order to elucidate if those interactions were due to the crosslinking between the lysine residue and the glutamine residue or were due to other type of interactions like disulphide bonds, the SDS-PAGE was performed under reducing conditions (Figure 1b). The analysis of the electrophoresis gels under reducing condition showed lesser amount of streaking but greater number of peaks than non-reducing conditions; which was expected due to the inclusion of ME in the buffer extraction. The rupture of the disulfide bonds between the proteins by the reducing agent yielded shorter protein chains that were able to enter the gel decreasing the amount of protein that was retained in the origin of the stacking and the resolving gel in the absence of transglutaminase (Figure 1b).

The major protein bands in the rice sample appeared at MW 15.1, 22.3 and 32.7 kDa (Figure 1b). These values are close to the values reported by Steenson and Shate in the *Basmati* rice (14.5, 20.4 and 33.1 kDa) and by Villareal and Juliano in *Indica* rice (16, 25 and 38 kDa). [24,25] The major protein bands in the rice-pea protein blend showed about the same MW (Figure 1b), thus probably they came from the rice proteins.

In the presence of TG, the gels showed a decrease in the intensity of the majority of the bands. The protein retained on the top of the stacking and resolving gels increased in the presence of TG (Figure 1b).

The bands with MW 50.7 and 42.5 from pea protein isolate disappeared after the TG treatment (Figure 1b), which probably corresponded to the acidic polypeptides of the legumin. Larré et al. also observed that after treating pea proteins with TG part of the reaction products did not penetrate the gel, due to the presence of polymers covalently cross-linked. [26] The acidic polypeptides from native legumin are prone to participate in the polymerisation reaction and the basic polypeptides, which have

lower MW than the acidic polypeptides, remain almost unchanged during the enzymatic reaction. [27] The acidic polypeptides are hydrophilic and are mainly located at the periphery of the protein, while the basic polypeptides are buried in the centre of the structure. [28]

The crosslinking catalysed by the TG may be intermolecular or intramolecular. The increase in the protein retained on the top of the gels in the presence of TG confirms the intermolecular crosslinking that resulted in an increase in the molecular weight of the polymers. [5,6] The increase in the band intensity on the top of the stacking and resolving gels in the presence of the TG besides the decrease observed under non-reducing conditions indicated that the direct effect of TG was the crosslinking reaction between proteins, and a secondary effect was the formation of disulphide bonds. The formation of disulphide bonds due to the TG activity was also reported by Gujral et al. and Larré et al. [4,29] The crosslinking reaction may bring near the sulphur containing amino acids, making easier the formation of these bonds.

SDS-PAGE analysis of the protein fractions

The effect of the TG on each protein fraction, obtained from a sequential extraction with different solvents, was determined by analysing the electrophoresis pattern of each fraction. The major pea proteins were extracted in the glutelin and in the albumin-globulin fractions (Figure 2, lane 1 and 7), which agrees with the results obtained in the quantification of the protein fractions by Kjeldahl. The prolamin was the minor fraction in all the cases. The pea proteins are classified as albumins and globulins, being the two major globulins named as legumin and thevicilin. [15,30] The high amount of proteins extracted under the conditions used for the glutelins could be ascribed to a change in the solubility of the proteins during the production of the protein isolate, since it has been reported that the process of the obtention of a protein isolate can modify the properties of the proteins. [22,23]

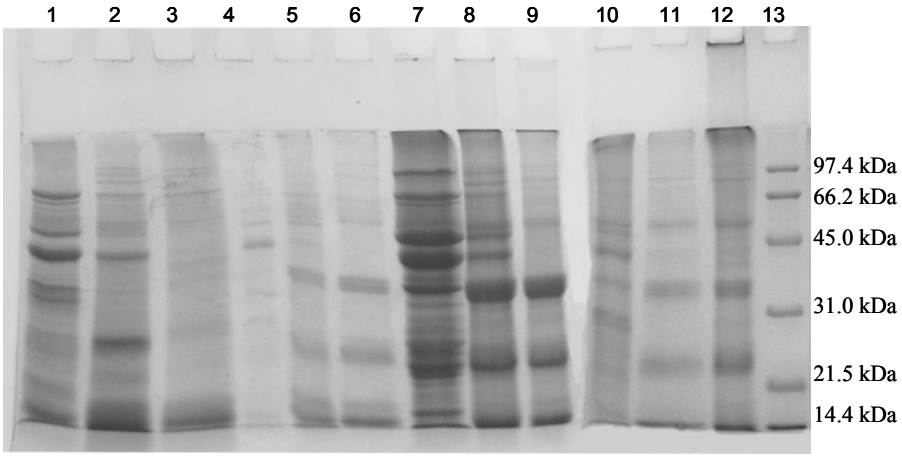

Figure 2. SDS-PAGE analysis of the protein fractions in pea protein isolate (without TG) (lanes 1, 4, 7, 10) and in rice-pea blend without (lanes 2, 5, 8, 11) and with TG (lanes 3, 6, 9, 12). Albumins-globulins (lanes 1, 2, 3), prolamins (lanes 4, 5, 6), glutelins step 1 (lanes 7, 8, 9), glutelins step 2 (lanes 10, 11, 12), molecular weight standard (lane 13).

In the presence of the TG, an evident decrease in the intensity of the bands in the albumin-globulin fraction was observed. In fact, almost all the bands of the rice-pea protein blend disappeared after the TG treatment. At the same time, an increase in the intensity on the top of the stacking and resolving gel was observed (Figure 2, lane 3). This result indicates the formation of protein polymers of higher MW with a concomitant disappareance of the lower MW polypeptides.

The addition of TG did not promoted significant changes in the prolamin fraction. In order to determine the nature of the interaction between the proteins induced by the TG, the glutelin fraction was extracted in two steps, firstly in the absence of ME and in the second step under reducing conditions in the presence of ME. The electrophoretic pattern was the same regardless the extraction conditions. The rice-pea protein blend showed two major bands in the glutelin fraction that appeared at the same MW than those of the rice sample (Figure 2, lane 8 and 9). This band would be the sum of rice and pea protein of the same molecular weight. This may be attributed to the

conservatism between the legume and cereal storage proteins, where similar sequences have been found. [31]

In the absence of the reducing agent (fist step), the intensity of the bands showed a pronounced decrease when TG was added, indicating the formation of large polypeptides unable to be extracted in those conditions (Figure 2 lane 9). Conversely, in the presence of the reducing agent, the addition of transglutaminase promoted an increase in the intensity of the bands, thus the rupture of the disulphide bonds favoured the extraction of the large polypeptides that remained in the precipitate. In addition, an increase in the intensity of the band on the top of the stacking and resolving gel was observed in the lanes of the glutelins in the presence of TG (Figure 2 lane 12). Again, this result confirmed no only the protein crosslinking but also the formation of disulphide bonds. Studies carried out on different proteins described that TG induces a decrease in the intensity or a disappearance of some protein bands and an increase in the protein material unable to enter the stacking or resolving gels, indicative of the intermolecular crosslinking between the proteins. [5,6,32-35]

Multistacking SDS-PAGE

From the analytical multistacking gel was obtained the proportion of proteins (relative IOD) retained in each zone of the gel that depended on their molecular weight (Table 2). The rice-pea protein blend sample showed a higher proportion of proteins with high molecular weight than the rice sample. It might be due to an association of either the pea and rice proteins or the pea proteins among them, obtaining aggregates with higher MW. This result is in agreement with the electrophoretic pattern of glutelin, where some band of rice-pea blend disappeared because they could not be extracted (Figure 2 lane 8). In the presence of the TG it was observed a decrease in the proportion of the protein retained in the 4% gel in both samples, rice and rice-pea protein blend, and in the 8% gel in the case of the

rice sample. This decrease could be attributed to the crosslinking reaction that produces polymers of higher molecular weight and more insoluble, unable to be extracted with the solvent used.

Table 2. Study of the effect of TG on rice and rice-pea blend by determination of the relative IOD of protein retained at various acrylamide concentrations (4, 8, 12%) of the analytical multistacking gel

	Relative IOD		
	4%	**8%**	**12%**
Rice	8.10	7.95	83.95
Rice+TG	2.83	2.27	94.90
Rice-pea blend	12.88	10.52	76.60
Rice-pea blend +TG	8.67	11.26	80.06

The Figure 3 shows the SDS-PAGE pattern of rice-pea proteins eluted from preparative multistacking gels (4, 8 and 12%). The same bands pattern was observed in the three zones of the gels (4, 8 and 12%). This result indicated that all the proteins participated in the formation of aggregates of higher MW. In the presence of the TG, the intensity of all the protein bands decreased and some of the bands even disappeared. The intensity on the top of the stacking and resolving gel in the lane corresponding to the 4% concentration increased (Figure 3 lane 5). Therefore, almost all the high molecular weight protein aggregates were involved in the crosslinking.

The transglutaminase activity induces the disappearance of numerous protein bands as a consequence of the formation of large protein polymers linked by isopeptidic bonds, but also some new disulphide bonds were formed as a secondary effect of the enzyme activity. The main protein fractions involved in those interactions

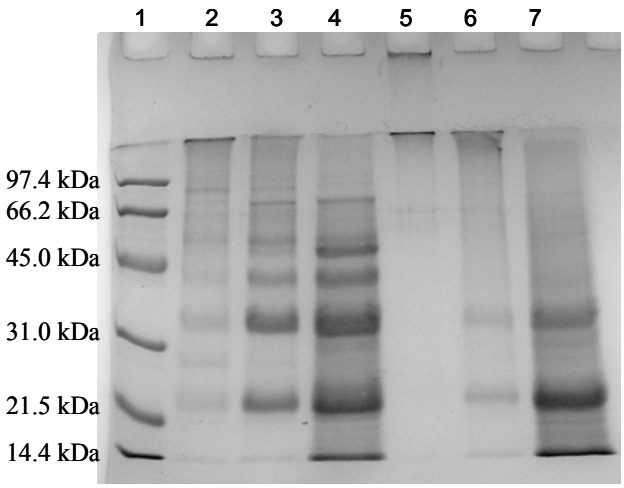

Figure 3. SDS-PAGE analysis of rice-pea blend from preparative multistacking gels. Concentrations of 4, 8 and 12% without TG (lanes 2, 3 and 4, respectively) and with TG (lanes 5, 6 and 7). MW standard (lane 1).

were the albumins and globulins from the pea protein isolate and rice flour and although in minor extent the glutelins were also crosslinked. Composite flours containing rice flour and pea protein isolate are proposed for obtaining a protein enriched rice dough with better amino acid balance and also a protein network formed of proteins aggregates of high molecular weight can be created in the presence of transglutaminase.

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Capítulo 4

Functional and rheological properties of protein enriched gluten free composite flours.

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Functional and rheological properties of protein enriched gluten free composite flours

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Abstract

Protein enriched composite flours comprising rice flour and soybean and pea protein isolates were made. Experimental design resulted in composite protein enriched blends with different functional, rheological, mechanical and surface related textural properties. The enzyme transglutaminase was added for reinforcing the protein network. Protein isolates induced a significant (p<0.01) increase in the water absorption of the composite blends, having also a synergistic effect and a decrease of the storage (G′) and viscous (G″) moduli. Protein isolates also modified the mechanical and surface related textural properties. Soybean protein isolate showed the most significant effect on the functional properties, rheometer and surface related textural responses. Crosslinking activity of the transglutaminase led to a significant decrease of the foaming activity and stability. Scanning electron micrographs of the composite blends showed that the usage of soybean, pea protein isolates and TG would be a promising approach to produce protein enriched blends for making fermented gluten free products.

Key words: rice flour, soybean, pea, proteins, transglutaminase, functional properties, rheology, microstructure.

INTRODUCTION

Yeasted bakery products addressed to coeliac patients require the development of complex matrixes with sufficient viscoelastic properties for holding the carbon dioxide released during the fermentation and enable to keep the structure during the expansion along baking. A considerable effort has been centred in the design of functional polymer blends that meet the technological requirements of gluten free fermented products. In fact, rice based breads have been successfully developed using several combinations of hydrocolloids like carboxymethylcellulose and hydroxypropylmethylcellulose (HPMC) (Ylimaki et al., 1991; Gujral et al, 2003). The use of HPMC also confers good quality properties to gluten-free bread based on 70% sorghum flour, and 30% potato starch (Schober et al., 2007). Crosslinking enzymes (transglutaminase and glucose oxidase) have been proposed as processing aids for improving gluten free bread quality (Gujral and Rosell, 2004a,b). Other different proposed combinations have been a complex formulation including corn starch, brown rice, soy and buckwheat flour. (Moore et al., 2004), or a mix containing different proportions of rice flour with corn and cassava starches obtaining gluten-free bread with uniform and an even distribution of cells over the crumb as well as a pleasant flavor and appearance (Sanchez et al., 2002; Lopez et al., 2004). In general, those polymer blends are based predominantly on carbohydrates, with a very low proportion of proteins. Scarce information is available about the use of enriched protein blends as gluten free matrixes.

Different protein sources can be added for improving the nutritional quality of gluten-free products, given that celiac disease in some cases leads to malnutrition. Dairy and soybean proteins are the most used proteins in gluten-free bread formulations (Gallagher et al., 2003; Ribotta et al., 2004; Moore et al., 2006; Marco and Rosell, 2008). Legumes are a good supplement for cereal-based foods since both legume and cereal proteins are complementary in essential amino acids.

Cereals are deficient in the essential amino acid lysine, while legumes have a high content of this amino acid. On the other hand, cereal proteins complement legume proteins in the essential amino acid methionine (Iqbal et al., 2006).

Soybean is highly used in Asian diet and nowadays its presence in Western diets is increasing due to the association of soybean protein consumption with lower risk of cardiovascular diseases (FDA, 1999). Besides, soybean is used in food technology for supplying desirable functional properties such as emulsification, fat absorption, moisture holding capacity, thickening, and foaming (Wolf, 1970). Although the most used legume protein is from soybean, pea proteins can also be successfully used in bakery products, obtaining a protein enriched product with better amino acid balance (Tömösközi et al., 2001). In addition, it has been reported that the addition of soybean or pea proteins to rice flour modified the mechanical properties of the rice-proteins blend dough, inducing a significant increase in the elastic modulus recorded by the oscillatory tests (Marco and Rosell, 2008).

The aim of this study was to design a protein enriched composite flour comprising rice flour and soybean and pea protein isolates. Transglutaminase, a transferase with crosslinking activity, was added for creating a protein covalent network. Functional, rheological and microstructure properties of the resulting protein enriched matrixes were determined.

MATERIALS AND METHODS

Commercial rice flour, from Harinera Belenguer SA (Valencia, Spain), had moisture, protein, lipid and ash contents of 12.2, 8.1, 1.0 and 1.0%, respectively. Protein isolates (pea and soybean) were from Trades SA (Barcelona, Spain). Moisture, protein, lipid and ash contents of the pea protein isolate were 6.2, 90.6, 1.1, 4.8%, respectively, and 6.0, 91.2, 0.4, 4.8%, respectively, in the soybean protein isolate. Composition of the different ingredients was determined following the AACCI

Approved Methods (1995). Microbial transglutaminase of food grade (Activa™ TG) (100 units/g) was provided by Apliena, S.A. (Terrasa, Barcelona, Spain). All reagents were of analytical grade.

Rice dough preparation

Rice flour was replaced by combinations of proteins and transglutaminase following a central composite design for sampling (Table 1). Design factors (quantitative independent factors) tested at five levels (-1.68, -1, 0, +1, +1.68), included soybean protein (from 1 to 25g/100g composite flour), pea protein (from 1 to 25g/100g composite flour) and transglutaminase (from 0.1 to 1.5g/100g composite flour). The model resulted in 17 different combinations of composite flours prepared in a Brabender farinograph (Duisburg, Germany) bowl (50g flour capacity) by mixing for 15 min all the ingredients. All the composite doughs were prepared at constant consistency. Dough was freshly prepared for the dynamic and static rheological properties. Whereas for determining the functional properties, composite flour doughs were freeze dried till further analysis.

Table 1. Central composite design for sampling.

Factors	Symbols	Coded-variable levels				
		-1.68179	-1	0	+1	+1.68179
Soybean protein (g/100)	SP	1.0	5.9	13.0	20.1	25.0
Pea protein (g/100)	PP	1.0	5.9	13.0	20.1	25.0
Transglutaminase (g/100g)	TG	0.1	0.4	0.8	1.2	1.5

Design factors are soybean protein isolate (SP), pea protein isolate (PP) and transglutaminase (TG). –1.68179, -1, 0, +1 and +1.68179 indicate coded levels of design factors. Axial distance: 1.68179. Values are expressed as g/100g of composite flours.

Oscillatory measurements

Dynamic rheological measurements of the dough were determined on a controlled stress rheometer (Rheostress 1, Termo Haake, Germany). The measuring system consisted of parallel plate geometry (rough plate 35 mm diameter, 1 mm gap). Rice dough, placed between the plates, rested for five minutes before starting the test. The rim of the sample was coated with Vaseline oil in order to prevent evaporation during the measurements that were performed at 30 ºC (Gujral et al., 2004a). Stress sweeps at 1 Hz frequency were carried out to determine the linear viscoelastic zone. Frequency sweep tests were performed from 0.01 to 10 Hz to determine the storage modulus (G´), loss modulus (G´´) and loss tangent (tan δ) as a function of frequency. Two replicates of each measurement were made.

Mechanical and surface related texture properties

Dough machinability was assessed by assessing the texture profile analysis (TPA) and dough stickiness in a TA-XT2i texturometer as described Armero and Collar (1997) using the Chen & Hoseney cell. The primary textural properties were measured in the absence of dough adhesiveness by using a plastic film on the dough surface to avoid the distortion induced by the negative peak of adhesiveness (Armero and Collar, 1997; Collar and Bollaín 2004). The adhesiveness was measured without the plastic film. Parameters registered included: hardness, cohesiveness, resilience, springiness, gumminess, adhesiveness and stickiness (for detailed information about those parameters see Armero and Collar, 1997). Three and ten repetitions for the TPA parameters and stickiness were made, respectively.

Functional properties

Composite flour doughs previously freeze-dried and ground with a refrigerated micro-hammer mill (powder with a particle size not exceeding 250 μm) were used for determining the functional properties.

The emulsifying properties of the composite flour doughs, were determined by the method of Pearce and Kinsella (1978). To prepare the emulsion, 2 ml refined sunflower oil and 6 ml freeze-dried samples suspension (0.5%) in 0.1M phosphate buffer (pH 7.0) were shaken together and homogenized in a T18 Ultra Turrax (Wilmington, NC, USA) at 22,000 rpm for one minute at 20 ºC (Gujral & Rosell, 2004c). Aliquots of fifty microliters of the emulsion were taken at zero and 30 minutes and added to 5 ml of sodium dodecylsulphate solution (0.1%, w/v). The absorbance of the diluted solutions was read at 500 nm. The emulsifying activity was expressed as the absorbance measured at 0 min, and the emulsion stability was expressed as ES (%) = (Abs_{30min}/Abs_{0min})x100 (Ahn et al., 2005; Babiker, 2000). Four replicates of each measurement were made.

The foaming properties of the different doughs were determined following the method of Miller and Groniger (1976) with slight modifications. Fifty milligrams of freeze-dried sample were added to 5 ml 0.02 M phosphate buffer pH 7.0 and homogenized for 1 min at 18,000 rpm in a T18 Ultra Turrax (Wilmington, NC, USA). Then, the blend was transferred to a measuring cylinder to determine the volume at zero and 60 min after homogenizing. Foaming capacity (FC) and foam stability (FS) were determined. The foaming capacity was expressed as the increased volume of mixture after the homogenization. Foam stability was calculated as FS(%) = ($FC_{60min}/FC_{initial}$) x 100. At least two replicates of each measurement were made.

The protein solubility of the freeze-dried samples was determined at pH 4 and pH 6. Samples (0.2%, w/v) were suspended in 0.05 M acetate buffer (pH 4) or 0.05 M phosphate buffer (pH 6), and after vortexing for 10 min the turbidity was measured at

500 nm, following the method of Babiker (2000). Four replicates of each measurement were made.

Scanning electron microscopy

The structure of the composite flours was analysed by scanning electron microscopy (SEM). Fragments of the freeze-dried samples were mounted on aluminium specimen stubs using doubled tape and sputter-coated with 100-200Å thick layer of gold and palladium by Ion Sputter (Bio-Rad SC-500). Samples analysis was performed at an accelerating voltage of 10kV with a SEM Hitachi 4100 from the SCSIE department of the University of Valencia.

Statistical analysis

The analysis of variance for each functional and rheology characteristic (response) was conducted using Statgraphics V.7.1 program (Bitstream, Cambridge, MN), in order to determine significant differences among the factors combination. Analytical data were fitted to multiple regression equations using the desing factors as independent variables. For each response with significant differences response surface plots were generated from the regression equations by using the Statgraphics program. Response surface plots were obtained by holding the independent variable with least significant effect on the particular response at constant value and changing the other two variables.

RESULTS AND DISCUSSION

Analytical data obtained from the central composite design samples on functional and rheological responses were fitted to second-order polynomial models using added principles (design factors) as independent factors in order to estimate response surfaces of dependent dough variables. Experimental data were submitted

to the analysis of variance to determine the main effects of the protein isolates and the transglutaminase and their interaction.

Effect of protein isolates and transglutaminase on the farinograph water absorption of the composite blends

Regression coefficients of the added principles obtained from the stepwise regression fitting model and analysis of variance are included in Table 2. The value of the determination coefficient (R^2) indicated that the model as fitted explained 99.84% of the variability in the water absorption. The water absorption determined by the farinograph was significantly (p<0.01) affected by the amount of soybean and pea protein isolates. The higher the amount of protein isolate added, the higher the water absorption was (Figure 1). Besides, the addition of both proteins simultaneously produced a significant synergistic effect. The soybean protein showed greater effect than the pea protein (Figure 1). The addition of soybean protein at the maximum level tested (+1.68179) and pea protein at the minimum level tested (-1.68179) induced an increase in the water absorption of 84.9 %. While in the opposite side, when pea protein was added at highest level and soybean at the lowest, the increase in this parameter was 38.9 %. The addition of both protein isolates at the maximum level produced an increase of 149.7 %. The increase in the water absorption produced by the addition of protein isolate, might be related with their water holding capacity, which is about 2.7-2.8 g/g for pea protein isolate and 4.0-5.0 g/g for the soy protein isolates (Vose, 1980). The same effect has been observed when 20% soy flour was added to wheat flour (Ahn et., 2005).

The addition of the transglutaminase resulted in a positive quadratic effect on the water absorption. The crosslinking activity of transglutaminase induces the formation of protein polymers with greater water holding capacity (Wang et al., 2007).

Table 2. Regresión equation[a] coefficients and analysis of variance for dough functional properties.

Coefficient	Functional properties						
	water absorption (%)	emulsifying activity (AU)	emulsion stability (%)	foaming capacity (ml)	foam stability (%)	solubility pH 4 (AU)	solubility pH 6 (AU)
b_0	240.9	0.309	61.23	0.43	62.60	0.650	0.740
b_1	41.2 **	-0.054 **	-17.36 *	0.09 *	19.22 **	-0.237 **	-0.219 **
b_2	21.8 **	-0.004	-2.58	0.07 *	5.55 *	-0.092	-0.059
b_3	1.3	-0.030	2.66	-0.08 *	-11.21 **	0.083	0.110
b_{11}	0.4	0.026	16.43 *	-0.04	-11.35 **	0.070	0.084
b_{22}	-1.2	0.025	-6.01	0.01	-3.17	0.028	0.033
b_{33}	2.3 *	-0.002	-2.48	-0.03	-12.23 **	-0.048	-0.018
b_{12}	3.3 **	-0.002	2.84	0.02	-3.97	-0.018	-0.063
b_{13}	1.5	-0.007	-5.71	0.03	8.09 *	-0.025	-0.071
b_{23}	1.3	0.005	-4.90	-0.01	-7.27 *	0.068	0.092
R-SQ	0.9984	0.8085	0.7404	0.8256	0.9600	0.8014	0.8069

[a] $y = b_0 + b_1x_1 + b_2x_2 + b_3x_3 + b_{11}x_1^2 + b_{22}x_2^2 + b_{33}x_3^2 + b_{12}x_1x_2 + b_{13}x_1x_3 + b_{23}x_2x_3$
where x_1 = SP, x_2 = PP, x_3 = TG.
AU: Absorbance units.
* Significant at $p<0.05$, ** significant at $p<0.01$.

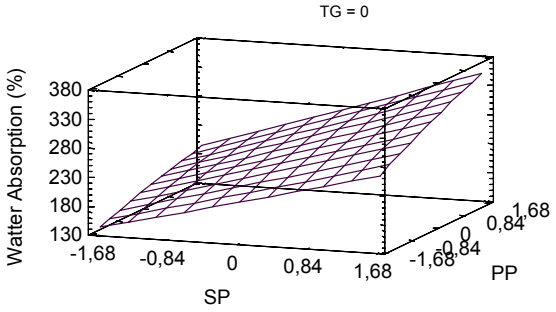

Figure 1. Response surface plots of water absorption . Effect of the addition level of the studied factors. SP: soybean protein, PP: pea protein, TG: transglutaminase.

Effect of protein isolates and transglutaminase on dynamic viscoelastic properties of rice flour

The viscoelastic properties of the rice dough containing different protein isolates were studied by dynamic oscillatory test. The mechanical spectra of all the samples showed storage or elastic modulus (G´) values higher than loss or viscous modulus (G´´) at all the frequency range tested, which suggest a viscoelastic solid behaviour of the doughs, which has already been described for rice based doughs (Gujral and Rosell, 2004a).

Data from the viscoelastic test were submitted to the analysis of variance to determine the main effects of the protein isolates and the transglutaminase and their interaction (Table 3). The value of the determination coefficients (R^2) indicated that the models as fitted explained 77, 76 and 98% of the variability in the G´, G´´ and tan δ, respectively. The additional level of soybean and pea protein isolates significantly affected the viscoelastic properties of the composite flour dough determined by the rheometer. The increase in the amount of soybean produced a significant linear

Table 3. Regresión equation[a] coefficients and analysis of variance for dough rheometer parameters.

Coefficient	Rheometer parameters		
	G´	G´´	Tan δ
	Pa	Pa	
b_0	68,868.5	11,566.0	0.169
b_1	-14,776.5 *	-1,816.0 *	0.010 **
b_2	-1,219.6	203.1	0.010 **
b_3	3,065.4	515.1	-0.001
b_{11}	-2,641.1	-946.6	-0.006 **
b_{22}	-12,667.9 *	-2,170.1 *	-0.003 *
b_{33}	-11,183.0	-1,850.8 *	-0.001
b_{12}	-2,999.3	-870.1	-0.007 **
b_{13}	2,386.3	392.0	0.002
b_{23}	5,755.0	935.6	0.002
R-SQ	0.7650	0.7580	0.9800

[a] $y = b_0 + b_1x_1 + b_2x_2 + b_3x_3 + b_{11}x_1^2 + b_{22}x_2^2 + b_{33}x_3^2 + b_{12}x_1x_2 + b_{13}x_1x_3 + b_{23}x_2x_3$
where x_1 = SP, x_2 = PP, x_3 = TG.
*Significant at $p<0.05$, ** significant at $p<0.01$.

decrease in the storage (G´) and the loss (G´´) moduli. The same effect was induced by the pea protein but derived from a significant quadratic effect. Besides, the presence of both protein isolates, soybean and pea, produced a significant increase in the tan δ, due to their positive linear and negative quadratic effect on that response (Figure 2). The interaction between soybean and pea protein produced a significant antagonist effect in the tan δ. The addition of soybean or pea protein isolate at maximum level when the other protein isolate was at the minimum level led to an increase in the tan δ of 77.9 and 77.7%, respectively (Figure 2). However, the

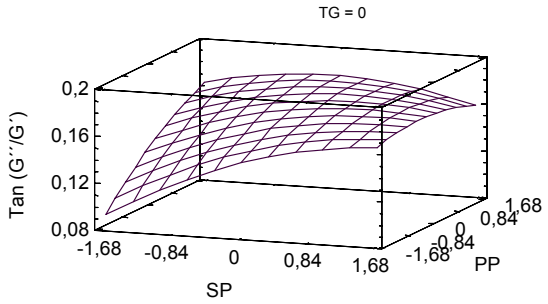

Figure 2. Response surface plot of tan δ determined by dynamic rheology. Effect of the addition level of the studied factors. SP: soybean protein, PP: pea protein, TG: transglutaminase.

addition of both proteins at the highest levels studied led to an increase in the tan δ of 72.2%.

Marco and Rosell (2008) observed a significant increase in G´ and G´´ when added 5% of pea or soybean protein to rice flour whereas the opposite trend was observed with the presence of egg albumen or whey protein. Differences can be attributed to the different water absorption applied in both studies, since constant water absorption (90%) was used in Marco and Rosell (2008), whereas the present study was carried out at constant dough consistency, adjusting the amount of water needed for obtaining dough with the same consistency. Results previously presented regarding the effect of pea and soybean proteins on the water absorption confirmed this assumption, since those proteins had a significant effect on the water absorption.

Transglutaminase only had a significant (p<0.05) negative and quadratic effect on the viscous modulus. Different findings have been reported pertaining the effect of the TG on cereal proteins. Some authors reported an increase in G´ values when cereal

proteins were treated with TG (Larré et al., 1998; Larré et al., 2000; Gujral et al., 2004a). Marco and Rosell (2008) also reported a significant increase in G´ when TG was added to rice flour enriched with 5% of different protein sources. In addition, some others described an increase in G´ and G´´ when wheat gluten solutions were treated with TG (Wang et al., 2007), but Truong et al. (2004) did not observe any difference in G´ and G´´ values after treating whey proteins with 0.12 unit/g of immobilized TG. However, the same authors observed a decrease of G´ when the TG concentration was increased; probably changes on the viscoelastic properties can be only observed after an extensive crosslinking of the protein. In the present study, a tendency to increase the storage and loss moduli and to decrease the tan δ values was observed when the level of TG increased, although the changes produced were not significant. Maybe, the great effect of the protein isolates is masking some of the changes produced by the TG.

Effect of protein isolates and transglutaminase on the mechanical and surface related textural properties of rice composite flours

Rheological assessment is a good indicator of polymer molecular structure and thus of end-use performance (Marin and Monfort, 1996). In the case of wheat dough, rheological analysis has been successfully applied as indicator of the molecular structure of gluten and starch, and as predictors of their functionality in breadmaking performance (Armero and Collar, 1997; Collar and Bollaín, 2005; Bollaín et al., 2006). Despite gluten free matrixes are structurally different than gluten dough, rheological assessment of the gluten free matrixes might give an indication of its further functionality. The experimental data obtained from the texturometer were statistically analyzed to determine the significance of the independent factors on the surface related textural responses of the protein enriched composite flours (Table 4).

Table 4. Regresión equation[a] coefficients and analysis of variance for mechanical and surface related textural responses of the composite flours.

Coefficient	Surface related textural responses						
	hardness	cohesiveness	resilience	springiness	gumminess	adhesiveness	stickiness
	N				N	N x m	force, N
b_0	47.2	0.209	0.062	0.854	9.8	0.518	0.121
b_1	-5.6 *	0.030 *	0.004	0.115 **	0.3	-0.088	-0.021 **
b_2	5.6 *	0.022	0.001	0.104 **	2.5 **	-0.006	-0.038 **
b_3	5.3 *	0.002	-0.006	0.024	1.5	-0.192 *	-0.016 **
b_{11}	4.0	-0.002	-0.002	-0.013	0.6	-0.094	-0.002
b_{22}	0.1	0.007	0.006	-0.036	0.6	0.078	0.010 *
b_{33}	0,3	0.017	0.007	0.011	0.9	-0.060	0.011 *
b_{12}	-1.0	0.022	-0.004	-0.105 **	-1.3	0.072	0.004
b_{13}	0.1	-0.011	-0.008	0.033	-0.4	-0.073	0.004
b_{23}	1.7	0.017	0.006	0.025	1.4	-0.060	-0.009
R-SQ	0.7970	0.7380	0.4000	0.9350	0.7920	0.7250	0.9610

[a] $y = b_0 + b_1x_1 + b_2x_2 + b_3x_3 + b_{11}x_1^2 + b_{22}x_2^2 + b_{33}x_3^2 + b_{12}x_1x_2 + b_{13}x_1x_3 + b_{23}x_2x_3$, where x_1 = SP, x_2 = PP, x_3 = TG.

* Significant at $p<0.05$, ** significant at $p<0.01$.

Mechanical and surface related texture parameters were dependent on the factors studied, soybean and pea protein isolate and TG, being particularly significant for hardness (R^2=0.7970), springiness (R^2=0.9350), gumminess (R^2=0.7920) and stickiness (R^2=0.9610).

The hardness was significantly affected by the level of addition of the three factors studied. The increase in the addition of soybean produced a decrease in the hardness, whereas the addition of pea protein or TG produced an increase in this parameter (Figure 3). The effect of TG on the hardness may be explained by the increase in the molecular weight of the proteins resulted from the crosslinking action of this enzyme, obtaining larger polymers (Marco et al., 2007, 2008). Wang et al. (2007) also observed higher values of hardness, adhesiveness, cohesiveness and chewiness in gluten gels containing TG than in those obtained without TG.

The cohesiveness was significantly (p<0.05) affected only by soybean protein content. The increase of cohesiveness promoted by soybean proteins agrees with the negative correlation between hardness and cohesiveness reported by Armero and Collar (1997) when studied wheat dough, because of harder dough experiments greater permanent damage to internal structure than less hard dough when both are exposed to the same strain. In wheat doughs, dough cohesiveness has been reported as a good predictive parameter of fresh bread quality, since more cohesive wheat doughs give softer breads with higher specific volume (Armero and Collar, 1997).

Both soybean and pea protein isolates produced doughs with higher springiness when the level of the protein increased (Figure 3). Conversely, the interaction between soybean and pea protein produced a significant (p<0.01) antagonist effect on the springiness.

Pea protein resulted in a positive linear effect on gumminess and the adhesiveness was only significantly reduced by the transglutaminase (Figure 3). The stickiness determined by the Chen & Hoseney cell was significantly (p<0.01) modified by the

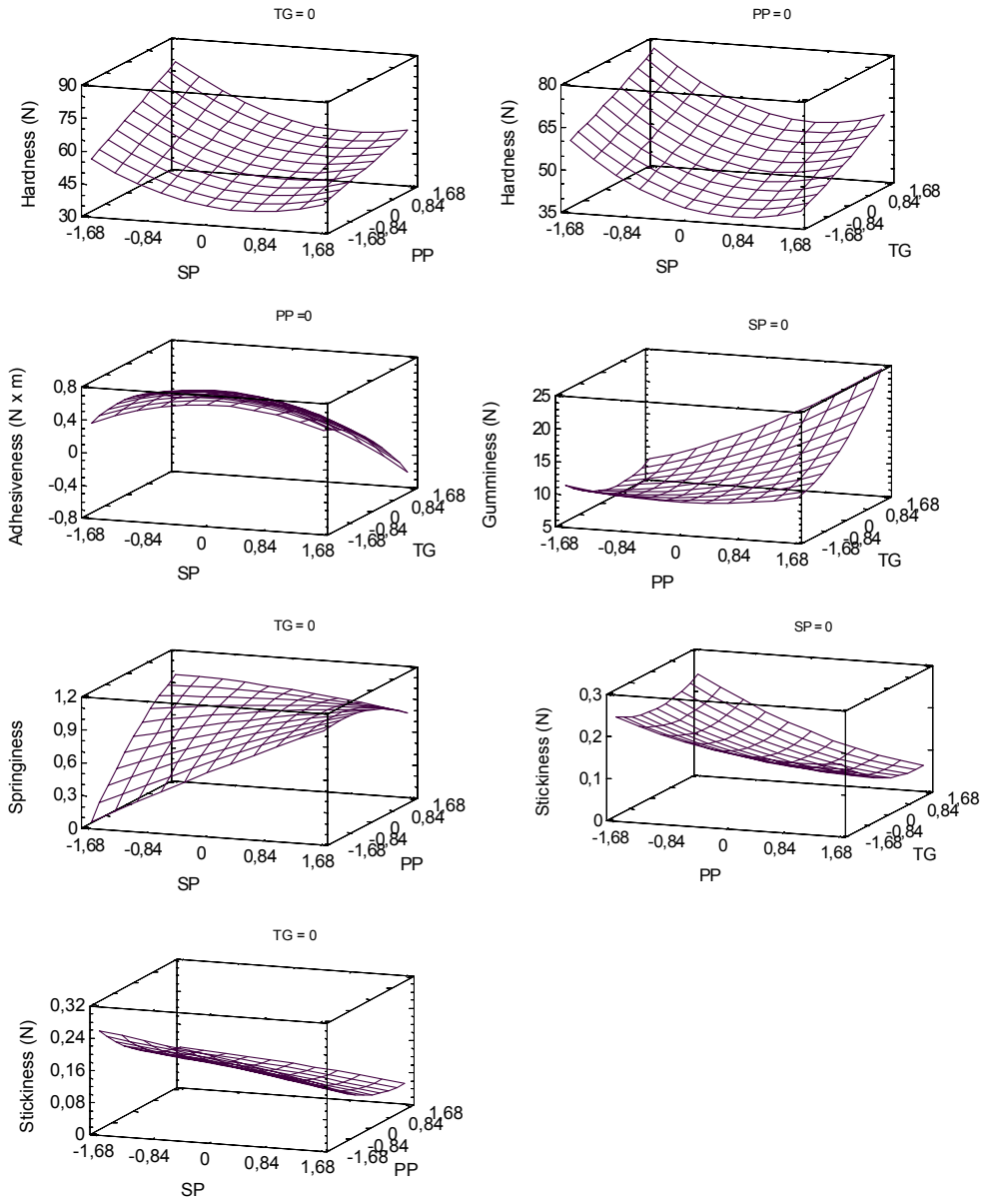

Figure 3. Response surface plots of the mechanical and surface related textural responses. Effect of the addition level of the studied factors. SP: soybean protein, PP: pea protein, TG: transglutaminase.

three factors. All of them decreased the stickiness of the doughs when the level of addition was increased (Figure 3). The stickiness showed a quadratic positive dependence on the addition of pea protein isolate and TG. The same effect of TG on dough stickiness has been already reported in wheat doughs (Tseng and Lai, 2002; Collar and Bollaín, 2004).

Functional properties of the composite blends

The value of the determination coefficients (R^2) indicated that the models as fitted explained 81 and 74% of the variability in the emulsifying activity and the emulsion stability, respectively (Table 2). The emulsifying activity of the composite blends was statistically affected by the level of addition of soybean protein isolate. An increase in the amount of soybean protein resulted in a decrease of this parameter. The emulsion stability was also significantly affected by the level of soybean protein isolate. The addition of this protein led to a negative linear and a positive quadratic effect on this response, as a consequence, the emulsion stability reached a minimum at 0.47 level of soybean protein (Figure 4). Therefore, that minimum should be avoided when looking for an increase in the emulsion stability. Soybean proteins shows higher emulsifying activity and emulsion stability than the pea proteins (Tömösközy et al., 2001). The addition of 20% soy flour to wheat produced a significant positive effect on the emulsifying activity of the samples (Ahn et al., 2005). Marco and Rosell (2008) reported that the addition of 5% of pea or soybean protein isolate to rice flour hardly modified the emulsifying activity of rice flour dough. These differences may be attributed to the different hydration of the composite blends, since water acts as a plasticizer defining the functional properties of the dough (Rosell and Marco, 2007). The effect of the TG in the emulsifying activity was almost statistically significant (p=0.0530). Siu et al. (2002) and Ahn et al. (2005) also observed a decrease in the emulsifying activity when cereal or soy proteins were treated with TG, which has been

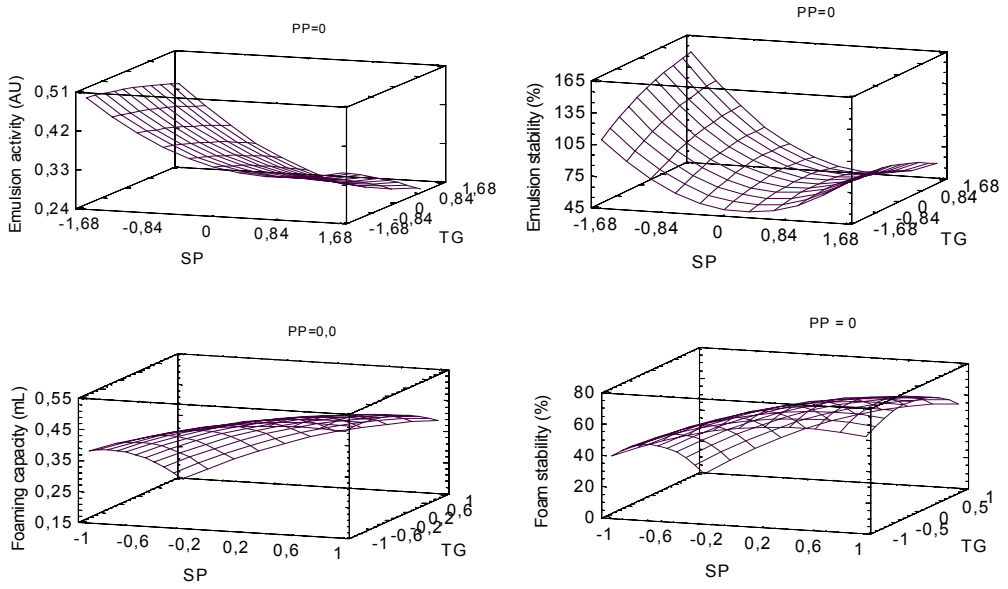

Figure 4. Response surface plots of functional properties. Effect of the addition level of the studied factors. SP: soybean protein, PP: pea protein, TG: transglutaminase.

attributed to the loss of solubility of crosslinked proteins. The increase in the molecular weight of polypeptide chains may lead to some loss of flexibility and reduces the protein ability to unfold at the oil-water interface (Siu et al., 2002; Marco et al., 2008 a,b). The presence of TG also induced an increase in the emulsifying stability, although that effect was no statistically significant (Table 2).

The regression models as fitted explained 83 and 96% of the variability in the foaming activity and the foam stability, respectively (Table 2). The foaming capacity and the foam stability were significantly affected by the addition level of the three factors studied in this experimental design. Both protein isolates, soybean and pea, showed a linear positive effect in these responses but the enzyme produced the opposite effect, an increase in the level of addition of TG resulted in a significant

decrease in these properties. Besides, the foam stability showed a negative quadratic dependence on the soybean protein and TG level (Figure 4). The interaction of the protein isolates with the TG was also significant in the foam stability. The interaction between soybean and TG had a synergistic effect, whereas the interaction between pea and TG showed an antagonist effect. Similar results regarding the effect of soybean on foaming activity and foam stabilty have been reported by Tömösközi et al. (2001). The decrease in the foaming activity and foam stability produced by the TG might be due to the increase in the molecular weight and the loss of the flexibility protein chains produced by the crosslinking activity (Marco et al., 2007, 2008).

The solubility of the composite blends enriched with proteins was studied at pH 4 and pH 6. The R^2 values indicated that the models as fitted explained 80 y 81% of the variability in the solubility at pH 4 and pH 6, respectively (Table 2). Soybean protein isolate was the unique independent factor that significantly affected the solubility at these pHs. In both cases, increasing soybean protein content produced a decrease in the solubility. The solubility obtained by the addition of soybean at the maximum level tested when the pea protein and TG were at the minimum value was 0.72 at pH 4 and 1.21 at pH 6. This agrees with the solubility profile of soybean given by Tömösközi et al. (2001) that showed higher solubility at pH 6 than at pH 4.

Microstructure analysis

The objective of the microstructure analysis was to elucidate the relationships between dough handling properties and food structure as suggested by Autio and Laurikainen (1997). Rice flour dough observed by scanning electron microscopy (SEM) showed a disrupted-like structure where starch granules were hold together by the proteins (Figure 5A). When transglutaminase (1%, w/w) was added, an uniform distribution of the starch granules through a more compact rice flour dough structure was observed (Figure 5B). Autio et al. (2005) and Bonet et al. (2006) observed an

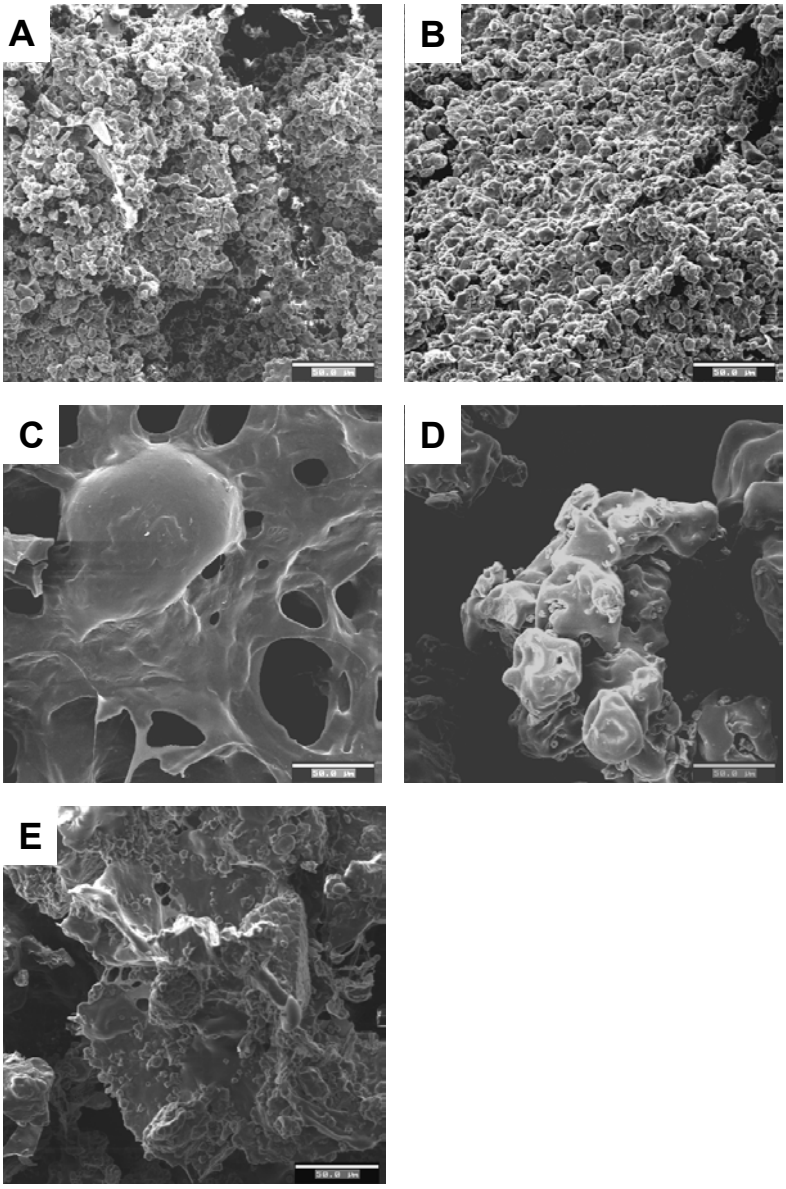

Figure 5. Scanning electron micrographs (x500) of different composite rice flour- proteins blends. A: hydrated rice flour, B: hydrated rice flour in the presence of 1% (w/w) TG, C: hydrated soybean protein isolate, D: pea protein isolate, E: composite blend containing SP, PP and TG at +1, +1, +1 levels, respectively.

enhanced protein network when analysed TG-treated wheat dough by scanning electron microscopy. Soybean proteins displayed a gel-like structure (Figure 5C), whereas pea proteins presented aggregates of a distorted spherical structures (Figure 5D). When all the independent factors (protein isolates and transglutaminase) were mixed together at (+1), (+1), (+1) coded levels, a composite protein enriched blend with a significantly affected microstructure was obtained (Figure 5E). Rice flour constituents and pea proteins seemed to be integrated in a compact structure surrounded by the soybean proteins, making difficult the differentiation between rice and protein isolate as independent structures. The effect of TG was not readily evident, although some protein strands were observed but it was not possible to decipher if they were from soybean proteins or consequence of the enzyme crosslinking. Increased aggregation of soy gels when treated with TG was reported by Fan et al. (2005), who analysed their structure using SEM. However, a continuous structure was observed without no longer differentiation between wheat and lupin independent protein structures, which might be attributed to the formation of heteropolymers between these two types of proteins (Bonet et al., 2006).

CONCLUSIONS

Composite protein enriched flours can be designed using rice flour, soybean and pea protein isolates. The addition of transglutaminase to the composite blends reinforced the network structure, although its effects were greatly masked by the high amount of protein isolates. Experimental design resulted in composite protein enriched blends with different functional, rheological, mechanical and surface related textural properties. Soybean protein isolate showed the most significant effect on the functional properties, rheometer and surface related textural responses. Scanning electron micrographs of the composite blends showed that the usage of soybean, pea

protein isolates and TG would be a promising approach to produce protein enriched blends for making fermented gluten free products.

ACKNOWLEDGEMENTS

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Capítulo 5

Breadmaking performance of protein enriched gluten free breads.

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Breadmaking performance of protein enriched gluten free breads

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Abstract

Soybean enriched, rice based gluten free breads were designed incorporating a structuring agent (hydroxypropylmethylcellulose, HPMC) and a processing aid (transglutaminase, TG). At dough level the effect of increasing amounts of soybean protein isolate (SPI), HPMC and water was studied in the Mixolab. Mixing and thermal characteristics showed the significant effect induced by water, soybean protein isolate, HPMC and TG, allowing the selection of the appropriate amounts for the breadmaking performance of enriched gluten free breads. The single addition or in combination of 4% HPMC, 13% soybean and 1% TG produced significant changes in the physical properties of the rice-based gluten-free breads. The presence of SPI blended with rice flour produced a significant decrease in the specific volume of the bread, although this detrimental effect was partially counteracted by its combination with HPMC, decreasing also the crumb hardness. The micrographs of the crumb showed the beneficial effect of the HPMC, obtaining a more open aerated structure. Protein enriched, gluten-free breads can be obtained with a combination of SPI, HPMC and TG.

Keywords: rice flour, soybean proteins, hydroxypropylmethylcellulose, transglutaminase, bread, gluten free.

INTRODUCTION

The ability of wheat proteins to develop a viscoelastic matrix is what makes wheat the most appropriate cereal for breadmaking. The protein fractions involved in the development of gluten are prolamins and glutelins that comprise 40 and 46% of the total proteins, respectively [1]. This viscoelastic matrix is able to retain the gas produced during the fermentation process, yielding an aerated crumb bread structure. However, gluten must be eliminated from the diet of celiac sufferers, because its ingestion causes serious intestinal damage. Rice flour is a gluten-free cereal frequently used for producing fermented products [2-4]. Rice flour has soft taste, is colourless, has low levels of sodium, and has easily digestible carbohydrates and low hypoallergenic properties. Nevertheless, rice flour is unable to develop a network with properties similar to gluten, likely due to its different storage protein ratio than the one found in wheat. In rice, the major storage proteins are the glutelins (65-85%) while prolamins are the minor fraction [5]. In order to overcome the problems associated with the lack of viscoelasticity different gums -hydroxypropylmethylcellulose (HPMC), locust bean gum, guar gum, carrageenan, xanthan gum, agar- and starches (corn, cassava and potato) are often incorporated in the recipes of fermented gluten free products [6-10]. Among the hydrocolloids used, the HPMC is one of the most appropriate to improve the volume and texture of the rice based, gluten-free breads [2, 11-14]. In addition, proteins from different sources such as soybean, egg albumen and dairy proteins can also be added to gluten-free flours in order to increase the nutritional value [15-18]. Soybean is a good counterpart for cereals, since legume and cereal proteins are complemented in the essential amino acids lysine and methionine improving the protein biological value of the product, [19]. Moreover, some health benefits are attributed to soy products, like the decrease of the risk for coronary heart disease associated with the consumption of soy protein due to the reduction in total low-density lipoprotein cholesterol and also in triacylglycerols [20]. However, high

levels of soybean flour may lead to a decrease in the specific volume and sensory properties of the bread [21]. Also a negative effect of high levels of soybean in wheat bread quality has been reported [22-23]. The deleterious effect of the addition of soybean has been associated with the lack of interaction between soy and gluten proteins [24]. However, the increase in the molecular size of the soybean proteins, produced by physical modifications leads to stronger soybean added wheat dough that yields larger loaf volumes [25]. Transglutaminase (TG) (protein-glutamine γ-glutamyltransferase, EC 2.3.2.13) is an enzyme whose most dominant reaction is the covalent crosslinking between proteins through the reaction between an ε-amino group on protein-bound lysine residues and a γ-carboxyamide group on protein-bound glutamine residues. Soybean has shown to be a substrate for the TG [26-29], obtaining protein chains with higher molecular weight that might improve the rheological properties of rice bread.

The goal of this work was to improve the rheological properties of soybean enriched, gluten-free doughs for making rice-based bread, by the addition of a structuring agent (HPMC) and a processing aid (TG), and to optimise the breadmaking process.

MATERIALS AND METHODS

Commercial rice flour, from Harinera Belenguer SA (Valencia, Spain), had moisture, protein, fat and ash contents of 12.2, 8.1, 1.0 and 1.0% (dry basis), respectively. The moisture, protein, lipid and ash contents were determined following the AACC Approved Methods no 44-19, no 46-13, no 30-25 and no 08-01, respectively (AACC 1995). Soybean protein isolate was from Trades SA (Barcelona, Spain). The protein isolate had moisture, protein, lipid, ash and carbohydrates (calculated by difference) contents of 6.0, 85.7, 0.4, 4.5 and 3.4%, respectively. Hydroxypropylmethylcellulose (HPMC) (Methocel K4M from Dow Chemical, USA) has

22.7% methyl groups and 11.2% hydroxypropyl groups. The HPMC viscosity of 2% solution in water was 4,664 mPa s at 20 °C. Microbial transglutaminase from Streptomyces spp. from Ajinomoto Co. Inc. (Tokyo, Japan) (100 units/g) was kindly supplied by Apliena, S.A. (Terrasa, Barcelona, Spain). All reagents were of food grade.

Mixolab measurements

Mixing and pasting behaviour of the rice flour dough and blends were studied using the Mixolab (Chopin, Tripette et Renaud, Paris, France), which measures in real time the torque (expressed in Nm) produced by passage of dough between the two kneading arms, thus allowing the study of its physico-chemical behaviour (Fig. 1). Rosell et al. [30] reported a detailed description of the equipment and the parameters registered. For the assays, 50 grams of rice flour were placed into the Mixolab bowl and mixed. The effect of variable amounts of water, soybean protein isolate, HPMC, and TG was tested using the Mixolab. The settings used in the test were 8 min for initial mixing, temperature increase at 2.3ºC/min until 90ºC, 7-min holding at 90ºC, temperature decrease at 4ºC/min until 50ºC, and 5-min holding at 50ºC; and the mixing speed during the entire assay was 80 rpm. Parameters obtained from the recorded curve give information about the protein stability subjected to mechanical and thermal constraints and both the gelatinization and gelling of starch. The following were the parameters obtained from the recorded curve (Fig. 1): initial consistency (C1), stability (min) or elapsed time at which the torque produced is kept constant, minimum torque (Nm) or the minimum value of torque produced by dough passage subjected to mechanical and thermal constraints (C2), peak torque (Nm) or the maximum torque produced during the heating stage (C3), the minimum torque during the heating period (Nm) (C4) and the torque obtained after cooling at 50°C

(C5) (more information about recorded parameters in Bonet et al. [28]; Rosell et al. [30] and Collar et al. [31]).

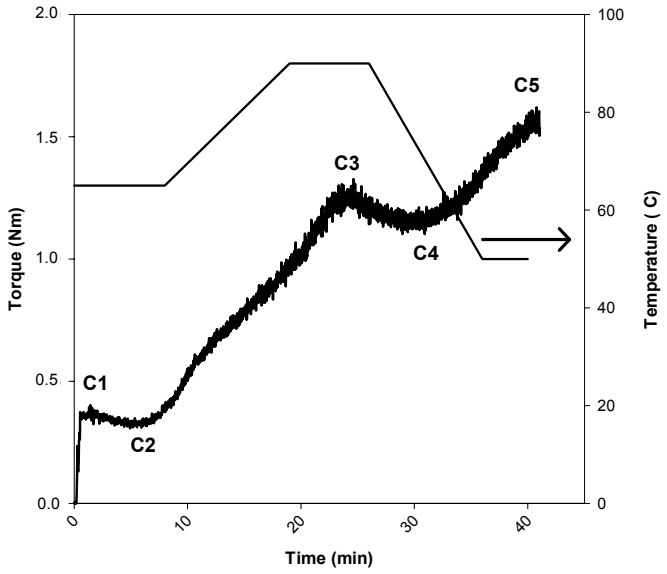

Fig. 1 Typical curve obtained in the Mixolab showing C1, C2, C3, C4 and C5 related to maximum dough torque, protein weakening, starch gelatinisation, starch breakdown and starch retrogradation, respectively.

Breadmaking process

The formulations used are showed in Table 1. Five hundred grams of rice flour and 550 mL of boiling water were mixed for 5 min in a 1-kg arm mixer. The dough was let to rest till the temperature decreased to 30 ºC. Then, the rest of the flour (500 g) and the other ingredients, and water (550 mL) were added and mixed for 5 min. Dough pieces of 400 g were put in well-greased pans and proofed for 60 min at 30 ºC and 85% RH. The dough pieces were baked for 1 hour at 175 ºC. Then, the loaves were

removed from the pans and cooled at room temperature for 1 h. Loaves packaged in polypropylene bags were stored at 24 ºC for 24 h and then used for bread quality assessment.

Table 1. Formulation of the gluten free breads.

	Rice flour	SPI	HPMC	TG
Rice	100			
Rice+SPI	87	13		
Rice+HPMC	100		4	
Rice+TG	100			1
Rice+SPI+HPMC	87	13	4	
Rice+SPI+TG	87	13		1
Rice+HPMC+TG	100		4	1
Rice+SPI+HPMC+TG	87	13	4	1

Values expressed in % (w/w) (rice-protein blend basis).
SPI: soybean protein isolate, HPMC: hydroxypropylmethylcellulose, TG: transglutaminase.

Bread quality assessment

In order to determine the bread quality, the volume (rapeseed displacement), weight, height/width ratio of the slices, crust and crumb colour, and moisture content were quantified. Crust and crumb colour were determined using a colorimeter (Chroma Meter CR-400/410, Konica Minolta, Japan). Moisture content was determined following the ICC Method. Besides, a texture profile analysis (TPA) of the bread crumbs was performed by a Texture Analyzer TA-XT2i (Stable Micro Systems, Surrey, UK). A bread slice of 1-cm-thickness was compressed up to 50% of its original height at a crosshead speed of 1 mm/s with a cylindrical stainless steel probe (diameter 25 mm). Measurements were performed after 24 h of baking. Values were the mean of four replicates.

Statistical analysis

Multiple sample comparison was used for the statistical analysis of the results, which was performed by using Statgraphics Plus V 7.1 (Statistical Graphics Corporation, UK). Fisher's least significant differences (LSD) test was used to describe means with 95% confidence.

SEM analysis

The structure of the crumb bread was analysed by scanning electron microscopy (SEM). Samples were freeze-dried and then mounted on metal stubs and sputter-coated with 100-200 Å thick layer of gold and palladium by Ion Sputter (Bio-Rad SC-500). Analysis of the samples was performed at an accelerating voltage of 10 kV with a SEM Hitachi 4100 from the SCSIE Department of the University of Valencia.

RESULTS AND DISCUSSION

Effect of the different ingredients and processing aids on the rice dough consistency

Mixing characteristics are strongly related to dough rheological properties, and they can be recorded as torque versus time curves obtained from small-scale mixers [32]. It is well known that the presence of biochemical constituents like the added ingredients, additives, and technological aids in dough formulation modify wheat dough characteristics, influencing baking performance [33-34]. In order to make a preliminary assessment of the individual effect of water content, HPMC, soybean protein isolate and transglutaminase on the breadmaking performance of rice flour, blends were subjected to a dual mechanical shear stress and temperature constraint using the Mixolab device. Information concerning mechanical and thermal protein weakening, starch gelatinization and starch gelling can be extracted from the recorded curves (Fig. 1) [30-31]. The effect on the dough rheological behaviour of the

addition of different amounts (65, 70, 75, 85 and 95%) of heated water (65ºC) to rice flour was determined by the Mixolab (Table 2). The purpose was to find out the maximum amount of water that could be absorbed by the flour constituents, since the plasticizer effect of the water is crucial in the breadmaking of gluten-free rice and low-protein starch breads [35-36]. The sample with 95% of water showed the lowest development time, which is the time to reach the maximum torque during the mixing stage. As was expected, the addition of increasing amounts of water produced a decrease in the maximum torque (C1) reached by the dough during the first mixing step. The highest decrease in the maximum torque or maximum consistency was observed when the added water increased from 65% to 70%, where the torque decreased from 1.45 to 0.7 Nm. The addition of 95% water produced a decrease up to 0.18 Nm. The increase in the amount of water added produced a decrease in all the recorded parameters, with the exception of the amplitude (dough elasticity), which showed a decrease with the increase in the water absorption but an inflexion point was detected at 85% water absorption. Amplitude defined as the bandwidth of the mixogram could be indicative of the role of water in the lubrication during mixing as it happens in the case of the mixograph and has been related to extensional properties of the dough during mixing [37]. A decrease in the minimum torque (C2), peak torque (C3), the minimum torque reached during cooling (C4) and the torque after cooling at 50ºC (C5) was also observed when increasing amounts of water were added, but this decrease was lower than the decrease observed during the mixing period previous to heating. The setback or the increase in the torque during the cooling (up to 50ºC) decreased when the amount of water increased. Further studies were carried out using 95% of water absorption because dough gave good amplitude and enough peak torque during heating, consequence of the starch gelatinization.

Table 2. Effect of water absorption on the rice dough characteristics during mixing and heating determined by using the Mixolab.

Water absorption (%)*	Development time (min)	Maximum torque (C1) (Nm)	Amplitude (Nm)	Stability (min)	Minimum torque (C2) (Nm)	Peak torque (C3) (Nm)	C4 (Nm)	C5 (Nm)	Setback (C5-C4) (Nm)	Cooking stability (C4/C3)
65	0.77	1.45	0.16	1.78	0.70	1.58	1.46	2.16	0.70	0.92
70	0.78	0.70	0.07	1.70	0.43	1.37	1.24	1.76	0.52	0.91
75	0.87	0.52	0.06	0.70	0.32	1.26	1.15	1.56	0.41	0.91
85	0.87	0.38	0.04	0.55	0.06	1.01	0.90	1.20	0.30	0.89
95	0.50	0.18	0.06	0.33	0.02	0.82	0.75	0.96	0.21	0.91

* Amount of water expressed on rice flour basis.

When the effect of the addition of increasing amounts of HPMC was tested (Fig. 2), a new consistency peak was detected before the gelatinization peak of the rice starch. The HPMC peak appeared at around 65ºC when greater amount than 2% was added. The HPMC is a hydrocolloid with a two-step gelation process. The first step involves the formation of a pregel that starts around 30ºC and the subsequent gelation produces a stable gel at around 75 ºC. The transition from pregel to gel of methylcellulose derivatives occurs at around 50ºC [38], but it seems that a change in the consistency is observed only at higher temperature. The maximum consistency of the dough increased with the addition of the hydrocolloid. The consistency of the doughs was higher in the presence of HPMC during the entire assay, but the difference was most noticeable during the mixing stage. The effect of the HPMC on the rice starch gelatinization decreased as long as the amount of hydrocolloid increased and simultaneously, a progressive increase of the HPMC gelation peak was observed. Finally, 4% HPMC was selected as the most convenient hydrocolloid amount because it gave enough consistency increase during mixing with a minor gelation peak of HPMC.

Soybean protein isolate was tested at two levels of rice flour replacement, 13 and 25% (flour-soybean proteins blend basis) (Table 3). The addition of the protein produced an increase in the development time, or the time necessary for hydrating all the compounds. The maximum consistency increased from 0.18 Nm in the absence of soybean to 0.75 or 1.34 Nm in the presence of 13 or 25% of soybean proteins, respectively. During the heating-cooling period, the effect of the soybean proteins was highly dependent on the amount of protein isolate present on the formulation, inducing opposite effects the presence of 13 or 25% of soybean proteins. The addition of soybean proteins increased setback, although this increase was higher with 13% than with 25% soybean. Bonet et al. [28] also described that an increase in the development time induced by the addition of protein sources (gelatin,

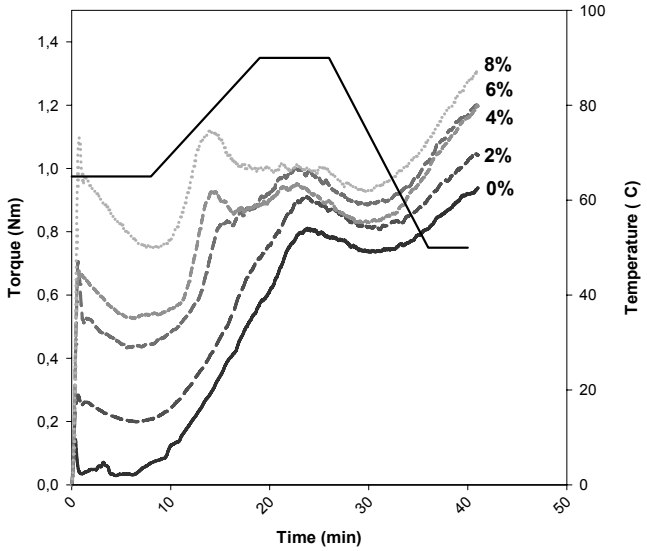

Fig. 2 Effect of the increasing amount of HPMC on the rice flour consistency determined by the Mixolab device.

egg and lupin) to wheat flour was attributable to the nature of the proteins, since proteins are the main components involved in water absorption. Regarding the peak torque, Bonet et al. [28] observed different effects depending on the protein source and the setback was significantly affected by the presence of soybean flour. The peak torque during heating was detected at around 65ºC, when soybean proteins were included in the formulation, which differed from the 90ºC found when only rice flour was tested. This behaviour was likely due to the soybean protein aggregation during heating treatment. Glycinin and conglycinin are the major proteins of soybean and both have the ability to form ordered gel structures. The behaviour of the commercial soybean protein isolates may be quite different from native soy proteins due to processing conditions causing denaturation and various states of aggregation [39].

Table 3. Effect of different ingredients and processing aid on the rice dough characteristics during mixing and heating determined by using the Mixolab.

Samples	Development time (min)	Maximum torque (C1) (Nm)	Amplitude (Nm)	Stability (min)	Minimum torque (C2) (Nm)	Peak torque (C3) (Nm)	C4 (Nm)	C5 (Nm)	Setback (C5-C4) (Nm)	cooking stability (C4/C3)
rice	0.50	0.18	0.06	0.33	0.02	0.82	0.75	0.96	0.21	0.91
rice + SPI- 13%	0.92	0.75	0.04	0.65	0.40	0.64	0.58	1.14	0.56	0.91
rice + SPI- 25%	1.30	1.34	0.09	2.52	0.99	1.23	1.00	1.31	0.31	0.81
rice + TG-1%	1.40	0.22	0.06	0.42	0.03	0.91	0.68	0.92	0.24	0.75
rice + SPI-13% + HPMC-4% + TG-1%	0.93	1.17	0.06	2.58	0.54	1.06	0.90	1.19	0.29	0.85
rice + SPI-13% + HPMC-4% + TG-1% (110% WA)	0.63	0.67	0.05	2.05	0.29	0.85	0.69	0.86	0.17	0.81

Assays were performed using 95% of water absorption (WA), unless otherwise stated.
SPI: soybean protein isolate, HPMC: hydroxypropylmethylcellulose, TG: transglutaminase.
Values expressed in % (w/w) (rice-protein blend basis).

The addition of transglutaminase only resulted in a increase of the development time and the dough stability (Table 3); the rest of the parameters recorded were similar to the ones observed in the rice flour.

The combined effect of HPMC, soybean protein isolate and TG was also studied (Table 3). HPMC was added at 4% level, soybean was added at 13% and TG at 1%. The addition of all the components gave doughs that showed a remarkable increase in the development time, and also in the values of stability and maximum torque during mixing. During the heating and cooling stages the increasing effect was not as notable as during mixing. This increase allowed increasing the addition of water till 110% (w/w, rice-protein blends basis). In those conditions, doughs with higher torque during the mixing stage compared to the rice dough were obtained. This combination was the one used for the breadmaking processes, although also the individual effect of the ingredients and processing aid studied were evaluated.

Effect of the different ingredients and processing aid on the quality of the gluten-free bread

The effect of the individual presence of soybean protein isolate, HPMC and TG was studied on the quality of gluten-free bread. The values obtained for crust and crumb colour, specific volume, height/width ratio of the slices and moisture content are showed in Table 4. The presence of soybean protein blended with rice flour produced a significant ($P < 0.05$) decrease in the specific volume of the bread; in contrast, the addition of HPMC produced an increase in the specific volume. The presence of TG did not modify the specific volume of the fresh rice-based bread. The detrimental effect of the soybean proteins on the specific volume was partially counteracted by its combination with HPMC. Therefore, the addition of HPMC allowed obtaining protein enriched, gluten-free breads with acceptable specific volume, similar to the one observed in the rice based bread.

Table 4. Effect of individual and combined addition of different ingredients (HPMC and soybean protein isolate) and processing aid (transglutaminase) on the quality of rice flour based breads.

	Specific volume (cm^3/g)	High/width ratio	Moisture content (%)	Crust color parameters			Crumb color parameters		
				L	a	b	L	a	b
Rice	2.00 B	0.08 b	45.59 e	45.86 c	5.60 a	19.20 b	54.47 a	-0.87 b	7.07 a
Rice+SPI	1.59 a	0.08 bc	46.07 f	44.25 c	9.22 c	21.06 c	61.25 bcd	0.61 cd	14.54 d
Rice+HPMC	2.71 c	0.12 e	41.66 a	47.41 c	5.51 a	18.93 b	65.24 d	-0.97 b	7.80 b
Rice+TG	1.97 b	0.08 b	46.13 f	30.96 ab	12.73 e	14.56 a	62.76 bcd	-1.22 a	8.82 c
Rice +SPI+HPMC	1.95 b	0.10 c	46.08 f	54.33 d	7.77 b	23.10 d	64.48 cd	0.55 cd	14.56 d
Rice+SPI+TG	1.57 a	0.07 a	45.43 d	28.33 a	13.70 f	14.17 a	59.70 b	0.77 d	14.52 d
Rice+HPMC+TG	2.69 c	0.11 d	43.70 b	33.92 b	11.26 d	14.53 a	60.86 bc	-1.09 ab	7.65 ab
Rice+SPI+HPMC+TG	1.96 b	0.08 bc	45.06 c	31.39 ab	13.21 ef	14.27 a	62.76 bcd	0.49 c	15.09 d

SPI (soybean protein isolate), HPMC (hydroxypropylmethylcellulose) and TG (transglutaminase) added at 13, 4, and 1% (w/w) (rice-protein blend basis), respectively.
Values followed by different letters in the same column are significantly different ($P<0.05$).

The loaves obtained with the addition of HPMC showed significantly higher height/width ratio of the slices. And that effect was reduced when soybean proteins were added in the formulation of the gluten-free bread. When all the components were added together, the values obtained for this parameter were similar to those obtained for the rice alone. The single addition of TG or soybean proteins did not produce any significant effect in this parameter. When soybean proteins and TG were added together a significant ($P < 0.05$) decrease was observed. Ribotta et al. [16] reported that rice-cassava based gluten-free breads with inactive soybean flour showed very dense crumb structure and low volume, whereas bread volume improved when active soybean flour was added. Also a negative effect on the bread volume was reported when soy products were added to wheat flour, where the soybean produced a loss of gas retention properties due to dough weakening and soy-interrupted gluten [40].

The improving effect of hydrocolloids on the bread volume has been reported by several authors [10, 12, 14, 41, 42]. The addition of hydrocolloids (carboxy-methylcellulose, agarose, xanthan, *β*-glucan, pectin) at 1% supplementation level produced an increase in the volume of gluten-free bread except for xanthan and pectin, although the opposite effect was observed when the hydrocolloid dosage increased from 1% to 2%, except for pectin [10]. McCarthy et al. [14] also reported a slight decrease in the gluten-free bread volume when increasing the addition level of HPMC. However, Gujral and Rosell [12] obtained higher volume when the addition level of HPMC to rice flour was increased until 4%, obtaining bread loaves with 2.5 cm^3/g specific volume. The improving effect of the HPMC on the bread volume might be due to their ability to retain water and also to the formation of a gel network during heating in the breadmaking process. This network will increase the viscosity and also

will give strength to the expanding cells of the dough; as a result, gas retention during baking improves, obtaining better bread volume [43].

The addition of HPMC produced a significant ($P < 0.05$) decrease in the moisture content of the bread, due to the ability of the hydrocolloids to retain water molecules. Opposite results were reported by Bárcenas and Rosell [41] when HPMC was added in the formulation of partially baked bread, because an extra amount of water was added to keep constant the dough consistency and compensate the water-retention capacity of the HPMC. In the present study, the reduction in the moisture content induced by the HPMC was partially masked when part of the rice flour was replaced by soybean proteins where rice starch molecules were replaced by protein molecules.

The bread colour also was affected by the addition of the studied components. The lower values of *L* (lightness) of the crust were obtained in the presence of TG. The breads containing TG had darker crust, which might be attributed to the maltose present in the commercial preparation of the TG that produces an increase in the non-enzymatic browning during the baking. The combination of HPMC and soybean produced the breads with the highest *L* value. The *a* scale varies from negative values (green hue) to positive values (red hue); and the *b* scale varies from negative values (blue hue) to positive values (yellow hue). The *a* values of the crust were all positive. The individual addition of HPMC did not promote any effect on the *a* parameter of the crust. But the single addition of soybean protein or TG or in combination with the other compounds produced an increase in this parameter, the effect of TG being higher than the one induced by the soybean. Presumably, the increase in the value of *a* obtained by soybean and TG were due to the increase of lysine groups and maltose, respectively, that are involved in the non-enzymatic browning reaction or Maillard reaction. The colour of the commercial soybean protein preparation also conferred some dark colour to the bread. The *b* values for the crust were positive (yellow hue) for all the samples. The presence of TG in the gluten-free

formulation yielded the lowest *b* values of the crust. The presence of soybean protein produced an increase in this parameter, and the crust bread with soybean and HPMC showed the highest *b* value.

Regarding the crumb, *L* increased when any of the components used in this study was incorporated to rice bread formulation. The *a* values were positive in the presence of soybean protein and negative in its absence. The single addition of HPMC or TG produced a decrease in this parameter. The *b* value increased in the presence of soybean (single or in combination). The addition of TG or HPMC produced a slight increase in this parameter.

The values obtained for the textural parameters of the rice bread are shown in Table 5. All the components incorporated significantly ($P < 0.05$) affected the hardness. The addition of HPMC produced a significant decrease of the crumb hardness. Conversely, the addition of soybean proteins induced a significant increase in the crumb hardness, only compensated when HPMC was included in the formulation. The hardness also increased in all the samples that contained TG, although to a lesser extent than the effect induced by the soybean proteins. The hardness increase resulting with the addition of TG has been reported previously in gluten-free, rice-based breads and it might be attributed to the protein crosslinking catalyzed by the TG, increasing the strength of the crumb [11]. The softening effect of the HPMC has been directly related to its effect on the specific volume of the bread [11-12, 41], because there is a positive relationship between the bread specific volume and the crumb hardness. The increase in the hardness due to the addition of soybean protein isolate agrees with the findings of Ahlborn et al. [35], who reported that the force needed to obtain compression values were higher for added-protein bread than for standard wheat bread.

Table 5. Texture profile analysis of the bread crumbs obtained from different rice based gluten-free formulations.

	Hardness (N)	Adhesiveness (N*m)	Springiness	Cohesiveness	Chewiness (N*m)	Resilience
Rice	7.25 b	-0.0019 a	0.773 a	0.577 bc	0.0032 b	0.222 c
Rice+SPI	22.32 e	-0.0047 b	0.862 bcd	0.595 c	0.0097 d	0.172 a
Rice+HPMC	1.95 a	-0.0014 a	0.905 cd	0.691 d	0.0012 a	0.259 d
Rice+TG	17.69 d	-0.0017 a	0.779 ab	0.511 ab	0.0070 c	0.214 bc
Rice+SPI+HPMC	13.12 c	-0.0222 d	0.844 abc	0.610 c	0.0067 c	0.189 ab
Rice+SPI+TG	26.76 f	-0.0077 c	0.819 abc	0.505 a	0.0111 e	0.168 a
Rice+HPMC+TG	3.34 a	-0.0028 ab	0.936 d	0.692 d	0.0022 ab	0.266 d
Rice+SPI+HPMC+TG	14.28 c	-0.0055 bc	0.840 abc	0.522 ab	0.0062 c	0.179 a

SPI (soybean protein isolate), HPMC (hydroxypropylmethylcellulose) and TG (transglutaminase) added at 13, 4, and 1% (w/w) (rice-protein blend basis), respectively.
Values followed by different letters in the same column are significantly different ($P<0.05$).

Regarding the adhesiveness (how the bread stuck to the palate, tongue, and teeth), the highest absolute value was obtained when soybean and HPMC were added jointly, but it decreased significantly in the presence of TG. A significant decrease in the cohesiveness (related to how the bread held together as it was masticated) was observed when soybean proteins and TG were added together, likely due to the hindering effect of the soybean proteins on the enzyme activity, which agrees with previous results of Ahlborn et al. [35] in protein-enriched wheat bread. Opposite results have been reported by Collar et al. [44], when TG was added to wheat breads. TG produced suitable effects on low-extraction rate flours (increased cohesiveness, volume, aroma intensity, typical taste and crumb cell ratio and decreased cell number), whereas the effects were adverse on high extraction rate flours (decreased volume, typical taste and crumb cell ratio and increased crumb hardness, chewiness and cell number). Regarding the resilience, it significantly decreased in the presence of soybean (single or in combination), while a significant increase in this parameter was observed when HPMC or HPMC+TG were added. The TG did not show any significant effect in the resilience.

Microstructure of gluten free bread crumbs

Scanning electron microscopy was used in order to see the effect of the addition of soybean protein isolate, HPMC and TG on the microstructure of the rice crumb bread. Rice crumb bread showed a large number of very small gas cells in a interrupted protein matrix, presenting a dense structure (Fig. 3a). In the presence of soybean was even more disaggregated, and the shape of the gas cells were very irregular (Fig. 3b). When TG was added, the structure of the crumb looked less disaggregated, showing less gas cells (Fig. 3d). This appearance might be attributed to the protein crosslinking that leads to a more continuous protein matrix. A more compact and homogeneous protein network due to the protein crosslinking was reported by Bonet

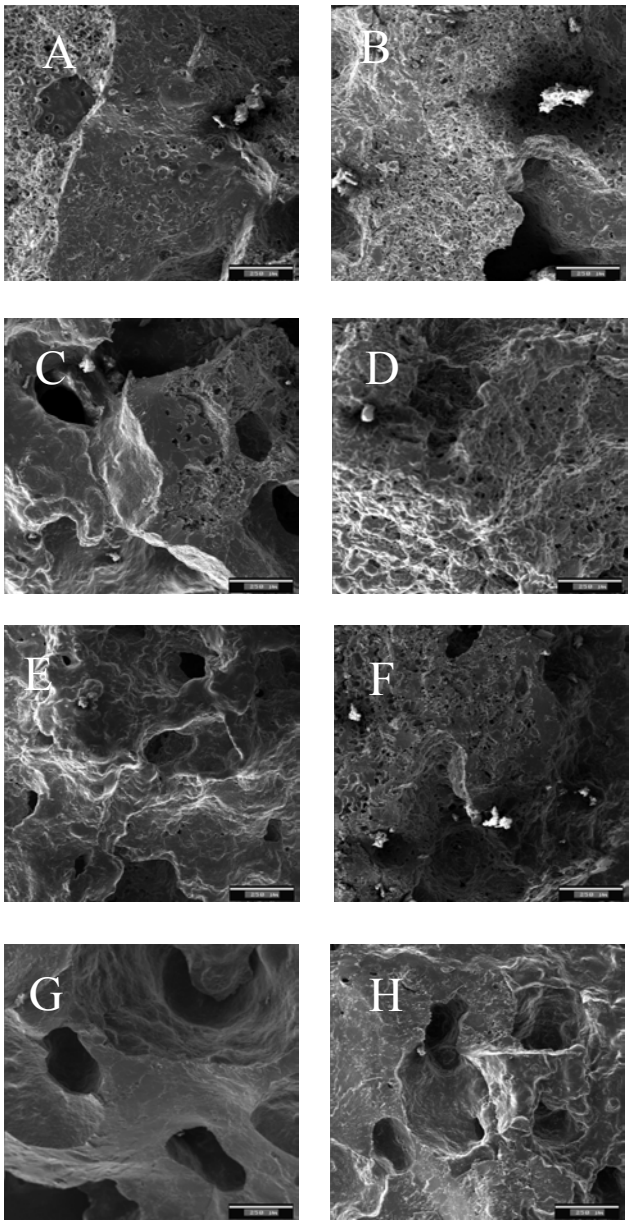

Fig. 3 Scanning electron micrographs (x100) of the crumb of different rice based breads. A: rice flour; B: rice flour + soybean protein isolate (SPI); C: rice flour + HPMC; D: rice flour + TG; E: rice flour + SPI + HPMC; F: rice flour + SPI + TG; G: rice flour + HPMC + TG; H: rice flour + SPI + HPMC + TG. In the formulation, SPI, HPMC and TG were used at 13, 4 and 1% (w/w), respectively.

et al. [28] in wheat doughs with soy flour when they were treated with TG. The addition of HPMC resulted in a more continuous matrix with improved structure, since less number and bigger size of cell gas were observed (Fig. 3c, e, g, h). The beneficial effects of the hydrocolloids on the dough or crumb structure have been previously reported in frozen doughs or partially baked bread [41, 45]. The HPMC seems to hold the constituents of the crumb covering them by a veil-like film [41], though also the physical interaction between the HPMC and the proteins or the starch granules could be possible [38, 46, 47]. In addition, the use of hydrocolloids, such as xanthan gum, in gluten-free bread produces a web-like structure similar to the structure of the standard wheat bread [35]. However, the hydrocolloids cannot produce this web-like structure when they are used in starch based gluten-free breads, indicating the importance of the proteins to form a continuous phase since the hydrocolloids alone do not seem to be enough to stabilize gas cells [35]. In the present study, the crumb of the bread containing HPMC and TG (Fig. 3g) and also soybean protein (Fig. 3h) showed the best structure, with more aerated structure.

CONCLUSIONS

The rheological properties of the soybean enriched-rice doughs can be modified by the use of structuring agents, such as HPMC, and processing aids, such as TG. The protein crosslinking produced by the TG reaction is reflected in a increase in the hardness of the bread crumb and a more continuous structure of the crumb observed in the micrographs. The use of HPMC allowed improving the volume of the bread, compensating the detrimental effect of the soybean, observing a more aerated structure closer to the wheat bread structure.

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Discusión General

La mayoría de productos derivados de cereales dirigidos a la población celíaca se caracterizan por un elevado contenido en hidratos de carbono (35-45%) a la par que un contenido muy bajo en proteínas (2,5-6%). El desarrollo de un pan sin gluten elaborado a base de harina de arroz y enriquecido en proteínas constituye una alternativa desde el punto de vista tecnológico y nutricional.

La elección del aislado proteico más adecuado para el desarrollo de productos panarios sin gluten basados en harina de arroz pasa por el análisis del efecto de estos biopolímeros sobre las propiedades funcionales y reológicas de la harina de arroz. El estudio sistemático realizado con aislados proteicos (soja, guisante, albúmina de huevo y suero lácteo) procedentes de distintos orígenes para determinar su efecto sobre la funcionalidad de masas de harina de arroz indica que las proteínas de origen vegetal tienen una mayor complementariedad funcional con las proteínas de cereales y mejoran el comportamiento viscoelástico (incremento en el módulo de almacenamiento o G′) de las masas obtenidas con harina de arroz. Resultados similares han sido obtenidos en masas batidas de maíz-trigo cuando se incorpora proteína de soja, de suero lácteo o albúmina de huevo, observándose efectos opuestos sobre la viscosidad de estas masas (Dogan, Sahin y Sumnu, 2005). Un refuerzo adicional de las características viscoelásticas de las proteínas se puede conseguir con la adición de la enzima transglutaminasa. En el caso concreto de las proteínas de cereales (trigo y arroz) la adición de transglutaminasa origina masas con mayor módulo de almacenamiento (Larré, Deshayes, Lefebvre y Popineau, 1998; Larré y col., 2000; Gujral y Rosell 2004a). En la harina de trigo, la transglutaminasa refuerza la red de gluten (Collar y Bollaín, 2004; Bonet y col, 2005). Este efecto se deriva del entrecruzamiento de las subunidades de glutenina de alto peso molecular que presentan un mayor contenido en lisina (Larré y col., 2000; Rosell y col., 2003).

El entrecruzamiento se ve reflejado en una disminución de las proteínas solubles debido a la formación de polímeros insolubles de alto peso molecular (Larré y col., 2000). Sin embargo, en las masas de harina de arroz se ven afectadas por el entrecruzamiento enzimático principalmente la fracción de las albúminas-globulinas. En el arroz los aminoácidos lisina, involucrados en la reacción de entrecruzamiento catalizada por la TG, están localizados mayoritariamente en la fracción de albúminas (Villareal and Juliano 1978), lo que explicaría la gran implicación de esta fracción en el entrecruzamiento catalizado por la TG. Cuando las proteínas de soja o guisante están presentes en las masas de harina de arroz, la transglutaminasa cataliza el entrecruzamiento entre las proteínas de la fracción de albúminas-globulinas, sin embargo su acción es más intensa sobre las proteínas solubles en las condiciones de extracción de las glutelinas (El-Moniem y col., 2000; FAO, 2007). La actividad de la TG origina polímeros proteicos de mayor tamaño y menor solubilidad, debido al entrecruzamiento intermolecular (Yildirim y Hettiarachchy, 1997; Basman, Koksel y Ng, 2002). Un efecto secundario de la reacción de entrecruzamiento catalizada por la TG es la formación de puentes disulfuro, ya que el entrecruzamiento conlleva un acercamiento de las cadenas proteicas facilitando la formación de estos enlaces (Larré y col., 2000; Gujral y Rosell, 2004a); este efecto secundario contribuye también al reforzamiento proteico. La acción directa de la transglutaminasa sobre las proteínas endógenas de la harina de arroz y las proteínas procedentes de los aislados proteicos, así como los puentes disulfuro generados originan una red polimérica que puede funcionar como matriz estructural en los productos libres de gluten.

El diseño de formulaciones libres de gluten en base harina de arroz y enriquecidas en proteínas pasa por realizar un diseño experimental que permita definir las concentraciones más adecuadas para optimizar las propiedades funcionales y

reológicas de las masas obtenidas. La adición creciente de proteínas conlleva un aumento en la absorción de agua debido a la capacidad de retención de agua de estas proteínas, que se sitúa en torno a 2.7-2.8 g/g en el caso de aislado de proteína de guisante y 4.0-5.0 g/g en el caso de aislado de proteína de soja (Vose, 1980). Este efecto también se ha observado al incorporar 20% de harina de soja a harina de trigo (Ahn y col., 2005). La adición de proteínas origina efectos opuestos sobre las propiedades mecánicas y de textura superficial de las masas, dependiendo de la naturaleza de las proteínas. Moore y col. (2004) atribuyen al alto contenido en proteínas de huevo, leche y soja, el incremento observado en la firmeza de masas libres de gluten. Sin embargo en el presente estudio se observa una menor dureza de las masas adicionadas con proteínas de soja, y el efecto contrario fue provocado por las proteínas de guisante. El reforzamiento proteico provocado por la TG se traduce en masas de harina de arroz más duras, lo cual coincide con el efecto descrito por Wang y col. (2007) en geles de gluten tratados con TG. Un aspecto a destacar es el aumento de la cohesividad de las masas de harina de arroz inducida por adición de proteína de soja, lo que concuerda con la correlación negativa entre dureza y cohesividad, que se ha definido como un índice predictivo de la calidad del pan de trigo, siendo recomendable mayor cohesividad de masas de trigo para obtener mayor volumen de pan fresco (Armero y Collar, 1997). Tanto las proteínas de soja o guisante como la TG modifican la microestructura de la masa, siendo más compacta por la acción de la TG. La adición conjunta de proteínas de soja y guisante no resulta una estrategia adecuada para el desarrollo de matrices panarias basadas en harina de arroz dado la ausencia de interacción entre ambas proteínas. El diseño de una formulación libre gluten basada en harina de arroz resulta más adecuado utilizando la proteína de soja, dada su mayor incidencia positiva en las características de la masa de harina de arroz.

A pesar del papel estructural que la proteína de soja reveló en los estudios de microscopía, es necesario la adición de algún ingrediente polimérico que contribuya y consolide la estructura reticulada necesaria en los productos sin gluten fermentados. Entre los hidrocoloides empleados en panificación para mimetizar las propiedades viscoelásticas del gluten en la masa de pan de trigo (Toufeile y col., 1994), el HPMC es uno de los más apropiados porque mejora el volumen y la textura de los productos sin gluten (Gujral y col., 2003; Gujral y Rosell, 2004a, 2004b; Sivaramakrishnan, Senge y Chattopadhyay, 2004; McCarthy y col., 2005).

El preparado formulado resultante de la combinación de harina de arroz con 13% (p/p, b.h.) de soja, 4% (p/p, b.h.) de HPMC y 1% de TG (p/p, b.h.) origina masas panarias con un buen comportamiento durante el amasado y una gran capacidad de absorción de agua. La adición de proteínas de soja en el pan de trigo, disminuye el volumen de las piezas de pan debido a la interferencia de la soja en la matriz de gluten, lo cual debilita su viscoelasticidad y capacidad de retención de gas (Ribotta y col., 2005). El mismo efecto se observa en los productos sin gluten (Moore y col., 2004), aunque este efecto negativo ha sido contrarrestado parcialmente con la adición de HPMC. Tanto la adición de TG como la de HPMC mejoran la microestructura de la miga de pan, obteniendo una matriz proteica más continua. Asimismo, en presencia de HPMC la miga presentó una estructura más abierta, mostrando un aspecto más similar al que se obtiene en pan de trigo. La formulación desarrollada origina un producto sin gluten con un aporte energético de 220,31 kcal/100 g de pan y cuyo perfil de composición (42,38% de hidratos de carbono, 10,56% de proteínas y 0,95% de lípidos) se asemeja al de los productos panarios con gluten.

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Conclusiones

- Las propiedades de la harina de arroz se ven modificadas de modo diferente por la adición de aislados proteicos en función de la naturaleza de las proteínas. El uso de transglutaminasa modifica el comportamiento viscoelástico de las masas de harina de arroz que contienen aislados proteicos, obteniendo diferentes tendencias dependiendo del origen de la proteína, siendo las proteínas de guisante y soja las que muestran un mayor valor del módulo de almacenamiento relacionado con el carácter elástico.
- La interacción entre las proteínas se intensifica mediante la formación de nuevos enlaces covalentes intermoleculares catalizados por la transglutaminasa y también por la formación indirecta de puentes disulfuro. Las fracciones proteicas más afectadas por la TG son las albúminas-globulinas en las masas de arroz y las masas conteniendo aislados proteicos (soja o guisante); y las glutelinas en las masas con aislados proteicos. El aumento de polímeros de alto peso molecular confirma el entrecruzamiento covalente intermolecular.
- Se pueden diseñar productos enriquecidos en proteínas, con un mejor balance en aminoácidos, mediante el uso de harina de arroz, proteína de soja y proteína de guisante con diferentes propiedades funcionales y reológicas en función de las proporciones utilizadas, siendo la proteína de soja la que produce mayores cambios en estas propiedades.
- Se puede obtener pan libre de gluten con alto contenido en proteínas mediante el uso de un agente estructurante como el HPMC y un coadyuvante como la TG. El entrecruzamiento de las cadenas proteicas origina una estructura de la miga de pan más continua. El uso de HPMC mejora el volumen del pan, compensando

los efectos negativos de la adición de altas concentraciones de proteína de soja, y permite el desarrollo de una miga con mejor alveolado.

- Se ha desarrollado un producto sin gluten basado en harina de arroz con un elevado contenido proteico (proteínas aportan el 19 % del valor energético del alimento) que puede considerarse como fuente de proteínas, y muy próximo al aporte mínimo del 20% requerido para definirse como alimento que posee alto contenido de proteínas según el DOCE L404/24 (30.12.2006), con unas características tecnológicas de calidad superiores a las de los productos sin gluten comerciales, el cual ha sido sensorialmente aceptado por un panel de consumidores constituido por las personas del grupo de investigación.