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Estabilidade estrutural e funcional de lectinas submetidas à irradiação gama: Potencial aplicação em alergia alimentar

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Á Marthyna
A minha irmã, Débora

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Resumo

Em alergias, apesar de sinais e sintomas típicos variados, o alérgeno proteico, através de seus epítopos lineares e conformacionais, é o principal responsável pela iniciação e manutenção da resposta alérgica inflamatória. A principal razão para o sucesso dessas proteínas na orquestração da alergia é a sua estabilidade estrutural, o que as tornam relativamente estáveis ao calor, aos extremos de pH, a proteases e a diversos métodos de processamento alimentar. Esta tese tem o objetivo de esclarecer o mecanismo pelo qual a irradiação de alimento atua sobre a estabilidade estrutural e funcional de alérgenos alimentares proteicos, e como esses conseguem proteger a sua estrutura enovelada nativa contra esse tipo de perturbação física. Os alérgenos selecionados para este estudo foram as lectinas: da semente da leguminosa *Cratylia mollis* (Cramoll), do feijão da leguminosa *Canavalia ensiformis* (Con-A) e a aglutinina do gérmen do trigo (WGA), visto que são proteínas bem estudadas e com estrutura resolvida, assim como suas vias de enovelamento-desenovelamento e desnaturação. Utilizamos técnicas espectroscópicas para investigarmos a relação estrutura-estabilidade dos alérgenos (lectinas), tanto na sua forma nativa quanto irradiada. Descrevemos, pela primeira vez, a desnaturação e fragmentação de alérgenos alimentares pela irradiação em alta dose sem o concomitante incremento de qualquer outro método de processamento. Mostramos também que os agregados formados apresentam suscetibilidade a agentes caotrópicos (ureia), através da dissolução dos agregados e formação de fragmentos peptídicos de baixa massa molecular. Verificamos que a conversão das formas nativas em agregadas envolve uma mudança substancial na estrutura terciária e na superfície hidrofóbica após irradiação, como foi visto por espectroscopia de fluorescência, Dicroísmo Circular (CD) e Calorimetria Diferencial de Varredura (DSC). Além dessas observações, vimos que o CD e a fluorescência fornecem evidências importantes para a inexistência de estados de glóbulos fundidos (molten globule) em alérgenos irradiados, uma vez que, em doses elevadas de radiação, a estrutura terciária, como também a estrutura secundária, não é mantida, o que indica a existência de precipitação em forma de agregados amorfos insolúveis com a falta de estruturas secundárias nativas. Posteriormente, averiguamos a resposta inflamatória alérgica de camundongos sensibilizados e submetidos a um desafio oral agudo e crônico com alérgenos irradiados ao analisar a perda de peso, o perfil de leucócitos, os níveis plasmáticos das citocinas IL-4, IL-5, eotaxina, RANTES e alterações morfológicas intestinais. Embora o procedimento de sensibilização não tenha induzido a perda de peso e alterações na contagem de leucócitos plasmáticos, a ingestão contínua de alérgenos não-irradiados reduziu o peso dos animais. Esses experimentos demonstram claramente uma associação entre a ingestão de lectinas não irradiadas, perda de peso corporal e eosinofilia. Tais efeitos alérgicos foram confirmados pela elevada secreção de eotaxina e exacerbado infiltrado linfocitário e eosinofílico no estroma das microvilosidades e na zona de cólon do jejuno proximal de camundongos sensibilizados. Contrariamente, alérgenos submetidos à alta dose de radiação e que precipitam em forma de agregados amorfos insolúveis revelaram valores significativamente mais baixos de eotaxina, e uma proporção de infiltrados linfocitários e eosinofílicos no estroma das microvilosidades muito menor, quando comparado a alérgenos nativos. Nestes termos, nosso estudo descreveu a relação entre a alergia e a modificação de alérgenos após a irradiação de alimentos. De fato, forneceu provas relevantes sobre a caracterização estrutural de proteínas irradiadas. Assim, acreditamos que a alta dose de

radiação pode tornar inócuo alérgenos alimentares proteicos de forma direta e irreversível. Nossos resultados destacam a necessidade de melhor caracterizar o envolvimento de alérgenos irradiados na modulação da resposta imune intestinal ao concentrar a atenção sobre os potenciais benefícios da irradiação de alimentos como um tratamento alternativo para abolir alergenicidade dos alimentos.
Palavras-chave: Alergia alimentar; Alérgeno alimentar; Irradiação de alimentos; Lectina.

Abstract

In allergies, despite a variety of signs and symptoms, the proteinic allergens, through its linear and conformational epitopes, is primarily responsible for the initiation and maintenance of the allergic inflammatory response. The main reason for the success of these proteins in the orchestration of the allergy is the balance provided by a complex three-dimensional structure, which makes them relatively stable to heat, extremes of pH, proteases and various methods of food processing. This thesis aims to clarify the mechanism by which food irradiation acts on the structural and functional stability of food allergen proteins, and how they can protect their native folded structure against this type of physical disturbance. The allergens selected for this study were lectins: Legume seed lectin *Cratylia mollis* (Cramoll), Bean lectin legume *Canavalia ensiformis* (Con-A) and wheat germ agglutinin (WGA), as they are well studied proteins, whose structure as well as their processes of folding-unfolding and denaturation have been elucidated. Spectroscopic techniques were used to investigate the structure-stability of allergens (lectins), both in its native and irradiated forms. For the first time the denaturation and fragmentation of food allergens by high-dose irradiation is described in isolation, without a concomitant increase of any other processing method. The aggregates formed were also shown to be susceptible to chaotropic agents (urea) through the dissolution of aggregates and formation of peptide fragments of low molecular weight. The conversion of native into aggregated forms was found to involve a substantial change in the tertiary structure and hydrophobic surface after irradiation, as seen by fluorescence spectroscopy, Circular Dichroism (CD) and Differential Scanning Calorimetry (DSC). In addition to these observations, the CD and fluorescence were found to provide important evidence for the absence of the fused globular state (molten globule) in irradiated allergens, since with high doses of radiation, the tertiary structure as well as the secondary structure are not maintained, which indicates the existence of precipitation in the form of insoluble amorphous aggregates with a lack of native secondary structures. Later, in order to ascertain the allergic inflammatory response of sensitized mice subjected to an oral dose of irradiated allergens, several physiological parameters were tracked: weight loss, leukocyte profile, plasma levels of IL-4, IL-5, eotaxin, RANTES, and histological changes to the intestines. Although the sensitization procedure did not lead to weight loss or changes in serum leukocyte count, the continuous ingestion of non-irradiated allergens reduced the weight of the animals. These experiments clearly demonstrate an association between ingestion of native allergens, weight loss and eosinophilia. Such allergic effects have been confirmed by high secretion of eotaxin and exacerbated eosinophilic and lymphocytic infiltrate in the stroma of microvilli and in the area of the proximal jejunum of the colon in sensitized mice. In contrast, allergens subjected to a high dose of radiation and that precipitate in the form of insoluble amorphous aggregates, showed significantly lower values of eotaxin and a much smaller proportion of lymphocytic and eosinophilic infiltrates in the stroma of microvilli when compared to native allergens. Importantly, our study described the relationship between the allergy and modification of allergens after food irradiation. In fact, this study provides evidence relevant to the structural characterization of irradiated proteins. Thus, we believe that a high dose of radiation can make proteinic allergens harmless in a direct and irreversible way. Our results highlight the need to better characterize the involvement of irradiated allergens

in the modulation of the intestinal immune response and focus attention on the potential benefits of food irradiation as an alternative treatment to abolish the allergenicity of foods.
Keywords: Food allergy, food allergen, food irradiation; lectin.

Lista de abreviaturas

BSA: Albumina de soro bovino

Bis-ANS: 4,4'-Bis (1-anilinonaphthalene 8-sulfonate)

Cramoll: Lectina da semente da leguminosa *Cratylia mollis*

Con-A: Lectina do feijão da leguminosa *Canavalia ensiformis*

CD: Dicroismo circular

CM: Centro de massa espectral

DSC: Calorimetria diferencial de varredura

HA: Atividade hemaglutinante

SDS–PAGE: Eletroforese em gel de poliacrilamida com SDS

SHA: Atividade hemaglutinante específica

RP-HPLC: Cromatografia liquida de alta resolução em fase reversa

RANTES: Regulador da secreção e expressão de células T após ativação

WGA: Aglutinina do gérmen do trigo

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1. Introdução

Desde os primeiros tempos, as pessoas procuram cuidar melhor de seus alimentos utilizando variados métodos de preservação, de modo a controlar a sua deterioração, a transmissão de doenças e a infestação de insetos. Essa degradação, causada por uma série de fatores, principalmente biológicos, mas também por fatores físicos e químicos, normalmente, modifica a qualidade do alimento, ao gerar, por exemplo, modificação na aparência (descoloração), odor desagradável e sabor inferior. Devido esses fatores, uma parte considerável da produção mundial de alimentos é desperdiçada por problemas de armazenamento, conservação e transporte. Ao desperdício, somam-se os custos sociais e econômicos das doenças causadas pela contaminação de alimentos.

Através dos séculos, as técnicas de preservação de alimentos foram se desenvolvendo com o aumento do conhecimento cientifico. A prevenção da deterioração dos alimentos através de práticas sanitárias adequadas e processamento apropriado têm sido a exigência de um mercado cada vez mais competitivo. Dentre essas várias alternativas disponíveis para indústria de conservação, a radiação ionizante tem sido eficiente ao melhorar nossa habilidade de conservar os alimentos e, ao mesmo tempo, reduzir a incidência de algumas doenças.

A irradiação de alimentos é um processo básico de tratamento comparável à pasteurização térmica, ao congelamento ou enlatamento. Este processo envolve a exposição do alimento, embalado ou não, a um tipo de energia ionizante. A energia radiante pode penetrar no alimento causando pequenas e inofensivas mudanças moleculares que também ocorrem no ato de cozinhar, enlatar ou congelar. De fato, a energia simplesmente passa através do alimento que está sendo tratado e, diferentemente dos tratamentos químicos, não deixa resíduo.

A tecnologia de irradiação de alimentos recebeu, durante os anos noventa, um grande interesse do público, da imprensa e da indústria alimentícia. O endosso do processo por entidades como o Departamento de Agricultura dos Estados Unidos (USDA) e a Associação Médica Americana (AMA), deram ao processo uma grande credibilidade junto aos consumidores. No Brasil, a legislação sobre irradiação de alimentos existe desde 1985 (Portaria DINAL no. nove do Ministério da Saúde, 08/03/1985). Entretanto, apesar desta

regulamentação, custos elevados e pouco interesse governamental têm restringido aplicação desta tecnologia de conservação em nosso país.

A alergia alimentar pode ser definida como uma reação adversa a um antígeno mediada por mecanismos fundamentalmente imunológicos. É um problema nutricional que vem apresentando um crescimento nas ultimas décadas, provavelmente devido à maior exposição da população a alimentos cada vez mais industrializados e complexos. Ele vem se tornando um problema de saúde em todo o mundo e está associado a um impacto negativo significativo na qualidade de vida.

Os alérgenos alimentares são geralmente proteínas, e a busca de métodos de processamento de alimentos para eliminá-los tem sido fruto de investigação a vários anos. Frequentemente, a desnaturação de proteínas e modificação de epítopos têm sido a opção mais prática. Entretanto, as oportunidades e os riscos envolvidos no uso de métodos de processamento no combate aos alérgenos alimentares tem sido superficialmente avaliado. Além disso, atualmente, pouco se sabe sobre como o processamento alimentar pode alterar alérgenos e, portanto, há uma necessidade de investigar sistematicamente a relação entre a alergenicidade e a modificação estrutural desses alérgenos.

Nesta tese, investigou-se como a radiação ionizante, um alternativo método de conservação, compromete a estrutura molecular de alérgenos alimentares proteicos, sua antigenicidade, e a resposta inflamatória alérgica de animais sensibilizados e submetidos a um desafio oral agudo e crônico. Para melhor compreensão do trabalho, da alergia, de alérgenos alimentares, do efeito do processamento alimentar, da irradiação de alimentos e dos processos de desnaturação/agregação proteicos, segue uma revisão bibliográfica apresentando contribuições científicas importantes que serão a base para a discussão e interpretação dos resultados apresentados.

2. Referencial teórico

2.1 Alergias alimentares

Apesar da maior conscientização e reconhecimento de doenças alérgicas por parte de médicos e pacientes, muitos pesquisadores acreditam que a incidência real de alergias alimentares aumentou substancialmente nas últimas décadas, similar ao aumento da prevalência de outras condições atópicas, como asma e rinite alérgica (Kagan, 2003). Embora dados epidemiológicos precisos sejam escassos, as estimativas da prevalência de alergia alimentar sugerem que, aproximadamente, 6-8% das crianças e 3-4% dos adultos têm reprodutíveis sintomas alérgicos (Sampson, 2005; Sicherer & Sampson, 2006). As alergias alimentares tornaram-se um grande problema de saúde e estão associadas a um impacto negativo significativo na qualidade de vida (Marklund et al., 2006). Esse risco ao bem-estar aumentou à medida que os alimentos consumidos em uma população têm sido cada vez mais processados e complexos (Taylor & Hefle, 2006).

2.1.1 Terminologia e classificação das reações adversas aos alimentos

A alergia alimentar é definida como uma reação clínica adversa após a ingestão de alérgenos, geralmente protéicos, presentes nos alimentos. Difere de outras reações adversas aos alimentos não imunomediadas que podem ser causadas por vários mecanismos, como deficiências de enzimas digestivas (no caso, por exemplo, de intolerância à lactose) ou toxinas (intoxicação alimentar por microorganismo patogênico), bem como aversões psicológicas (Ferreira & Seidman, 2007).

Em 2003, a Organização Mundial de Alergia (World Allergy Organization) propôs uma simples classificação das reações adversas aos alimentos com base no mecanismo patogênico (**Figura 1**). A hipersensibilidade passou a ser usada para descrever sintomas ou sinais reproduzíveis causados pela exposição a um estímulo definido em uma dose tolerada por pessoas normais. Por outro lado, a intolerância sugere uma resposta fisiológica anormal não imunomediada a um agente desencadeador. O termo atopia foi empregado para designar uma característica que torna um indivíduo suscetível ao desenvolvimento de várias alergias, enquanto que alergia é uma reação de hipersensibilidade desencadeada por uma

resposta imunológica que podem ser mediadas por IgE ou não (Johansson et al., 2004; Ferreira & Seidman, 2007).

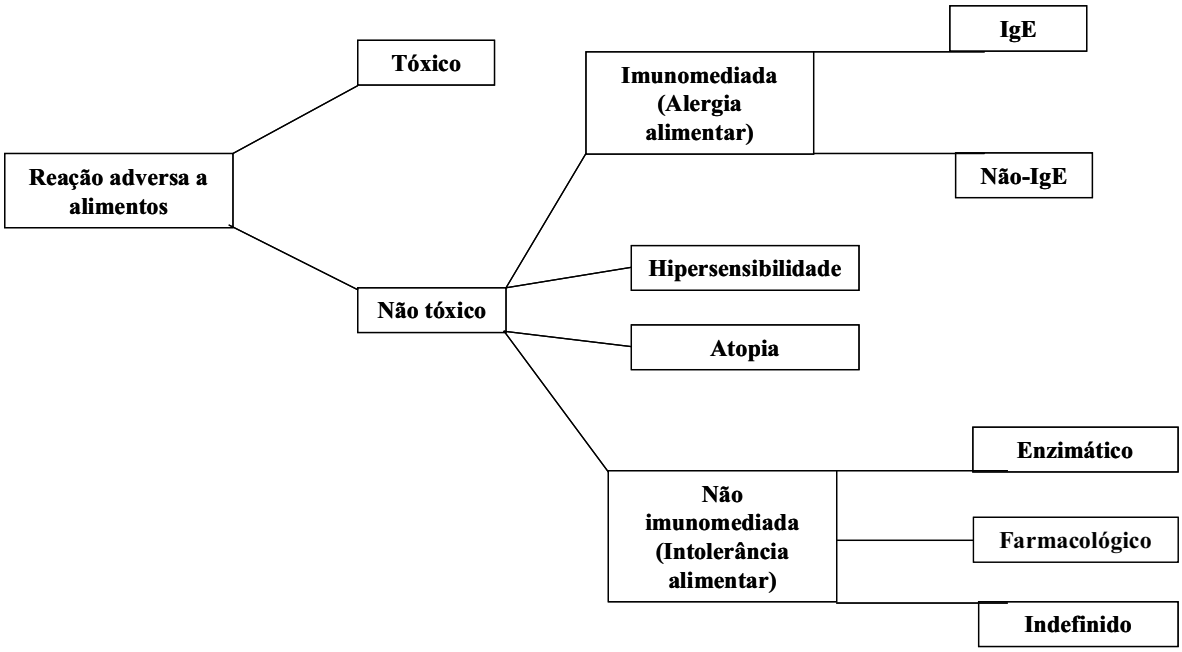


Figura 1. Reações adversas aos alimentos. Classificação segundo Organização Mundial de Alergia (Fonte: Johansson et al., 2004).

2.1.2 Sistema imune intestinal

2.1.2.1 Imunidade inata

O trato gastrointestinal abrange a maior área de superfície no corpo humano e é composta de uma simples camada de células que separa o ambiente externo dos órgãos internos estéreis. Sua função principal é processar o alimento ingerido em uma forma que possa ser absorvido, enquanto, ao mesmo tempo, impede a penetração de agentes patogênicos prejudiciais ao organismo (Brandtzaeg, 1998). A mucosa gastrintestinal consiste de componentes fisiológicos e imunológicos.

A barreira fisiológica inclui uma única camada de células epiteliais unidas por junções de oclusão (tight junction) e coberta por uma camada de muco espesso que retém partículas, bactérias e vírus. Além disso, enzimas, sais biliares, e os extremos de pH servem

para destruir os patógenos e tornar antígenos menos imunogênicos (Chehade & Mayer, 2005).

Macrófagos e neutrófilos têm sido as células efetoras mais importantes do sistema imunológico inato, mas há evidências de que outras células, como mastócitos e eosinófilos, também estejam envolvidos (Medzhitov & Janeway, 2000). Essas células identificam antígenos através de receptores de reconhecimento, como o receptor Toll-like (Wagner, 2001), e, assim, compõem a primeira linha de defesa imunológica do trato gastrintestinal.

2.1.2.2 Apresentação de antígenos, respostas imunes adaptativas e tolerância oral

Considerando que o sistema imune sistêmico elabora uma resposta inflamatória rápida quando confrontado com quantidades relativamente pequenas de um antígeno, o sistema imune intestinal regularmente encontra enorme quantidade de antígeno e a reatividade imunológica ao alimento e aos organismos comensais deve ser suprimida (i.e. desenvolve tolerância oral).

As células apresentadoras de antígeno (células epiteliais intestinais, as células dendríticas e células T reguladoras) da mucosa intestinal diferem de células apresentadoras sistêmicas devido à baixa expressão de moléculas coestimulatórias, tais como CD80 (B7-1) e CD86 (B7-2), as quais interagem com CD28 e outros receptores sobre células T (Rugtveit et al., 1997). Essa propriedade contribui para o estado normal hiporesponsivo do sistema imunológico intestinal, pois o complexo principal de histocompatibilidade de classe II, sem esses sinais coestimulatórios, preferencialmente induz anergia ou eliminação de células T (**Figure 2**).

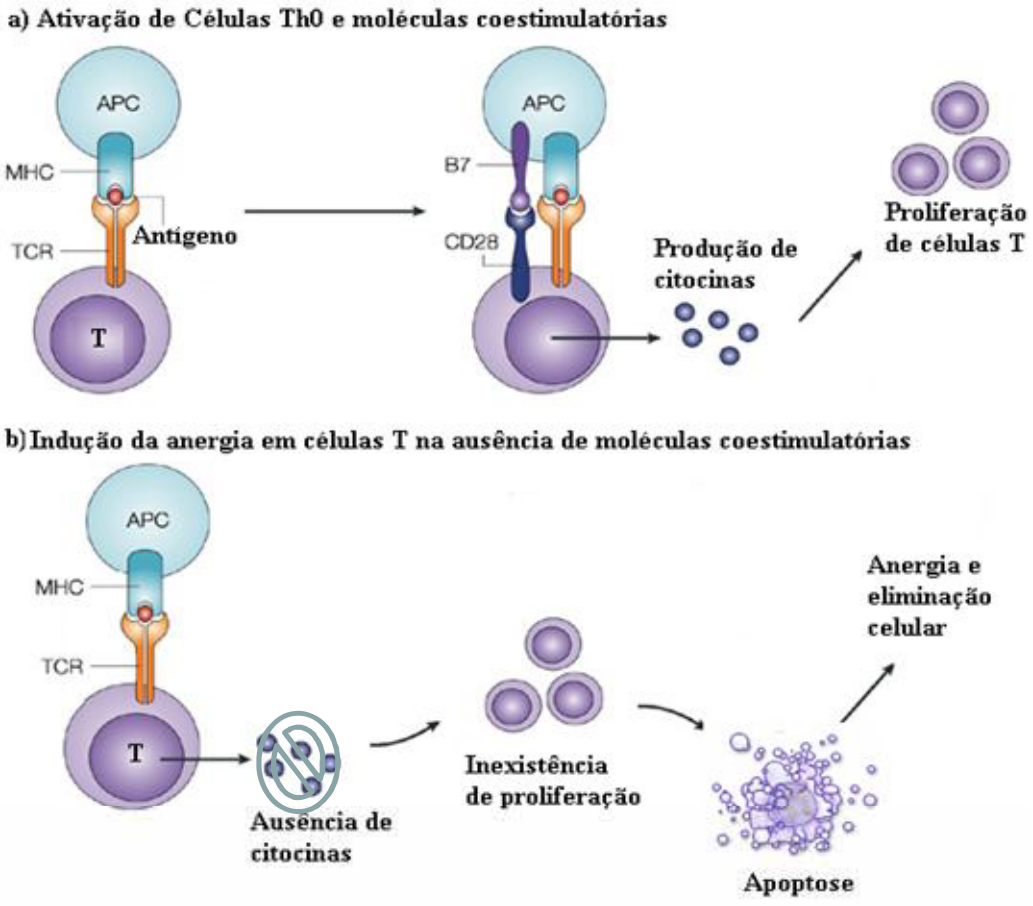


Figura 2. Estado hiporesponsivo do sistema imunológico intestinal. **a**) As células apresentadoras de antígeno (APC) sistêmicas através do complexo principal de histocompatibilidade (MHC) reconhece os antígenos, os quais participam da ativação de linfócitos (T) pela ligação aos receptores de células T (TCR) e moléculas coestimulatórias, tais como CD80 (B7-1) e CD86 (B7-2), que interagem com CD28. Essa ativação promove a liberação de citocinas responsáveis pela proliferação de células T. b) As células apresentadoras de antígeno da mucosa intestinal devido à baixa expressão de moléculas coestimulatórias, preferencialmente induz anergia ou eliminação de células T.

O sistema imune adaptativo com células e fatores (linfócito intraepitelial, lâmina própria, placas de Peyer, secreção de IgA, e citocinas) que também fornece uma barreira ativa ao antígeno em conjunto com células apresentadoras de antígenos desempenham um papel central no desenvolvimento da tolerância oral (Mowat, 2003; Strobel & Mowat, 2006). Apesar da evolução da barreira gastrintestinal, cerca de 2% de antígenos de alimentos ingeridos são absorvidos e transportados por todo o corpo em formas imunologicamente intactas, mesmo através do intestino saudável e maduro (Husby et al., 1985).

Fatores diversos podem influenciar o desenvolvimento de tolerância, tais como propriedades moleculares do antígeno e a dose e frequência de exposição. Estudos em modelos animais mostraram diferenças nas respostas imunes, dependendo da dose de antígeno ingerido: altas doses de tolerância envolvem eliminação de células T efetoras, e baixa dose de tolerância é o resultado da ativação de células T reguladoras (Chehade & Mayer, 2005). Estudos indicam que a flora intestinal de comensais também desempenha um provável papel na indução de tolerância oral, como inicialmente sugerido pela observação de que ratos criados em um ambiente livre de germes tem uma redução na tolerância oral (Sudo et al., 1997).

2.1.3 Distúrbios de tolerância alimentar

A inflamação alérgica intestinal requer uma quantidade elevada de alérgeno no lúmen intestinal para desencadear uma resposta proliferativa do sistema imunológico. Essa exposição ao antígeno é favorecida devido a alterações na barreira física, na imaturidade, ou distúrbios adquiridos do sistema de defesa, tal como em infecções entéricas. Fatores genéticos e ambientais podem também regular a permeabilidade do intestino e do tecido linfóide associado (**Figura 3**).

A inflamação não-específica induzida por bactérias, vírus ou toxinas pode predispor a uma perda da tolerância e ao desenvolvimento subsequente de hipersensibilidade imunológica. Isso ocorre porque as células dendríticas e epiteliais perdem a hiporeatividade e voltam a expressar moléculas coestimulatórias, as quais, finalmente, tornam-se capazes de estimular linfócitos T a produzir citocinas e células efetoras a produzir IgE (Eigenmann & Frossard, 2003). Células T alérgeno específicas podem ser isoladas de sangue, pele e mucosas em pacientes com alergia alimentar, e, caracteristicamente, elas expressam um fenótipo de células Th2 liberando citocinas, que desempenham um papel central na indução e manutenção da resposta alérgica por regular a síntese de IgE e a quimiotaxia de células inflamatórias (De Vries, 1998; Bischoff et al., 1999; De Vries et al., 1999; Hogan et al., 2002).

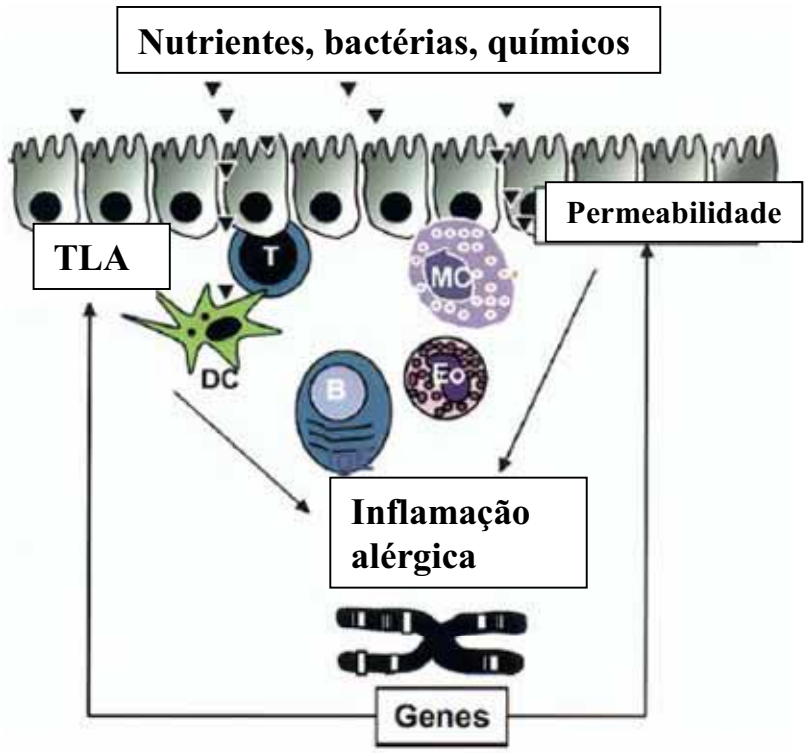


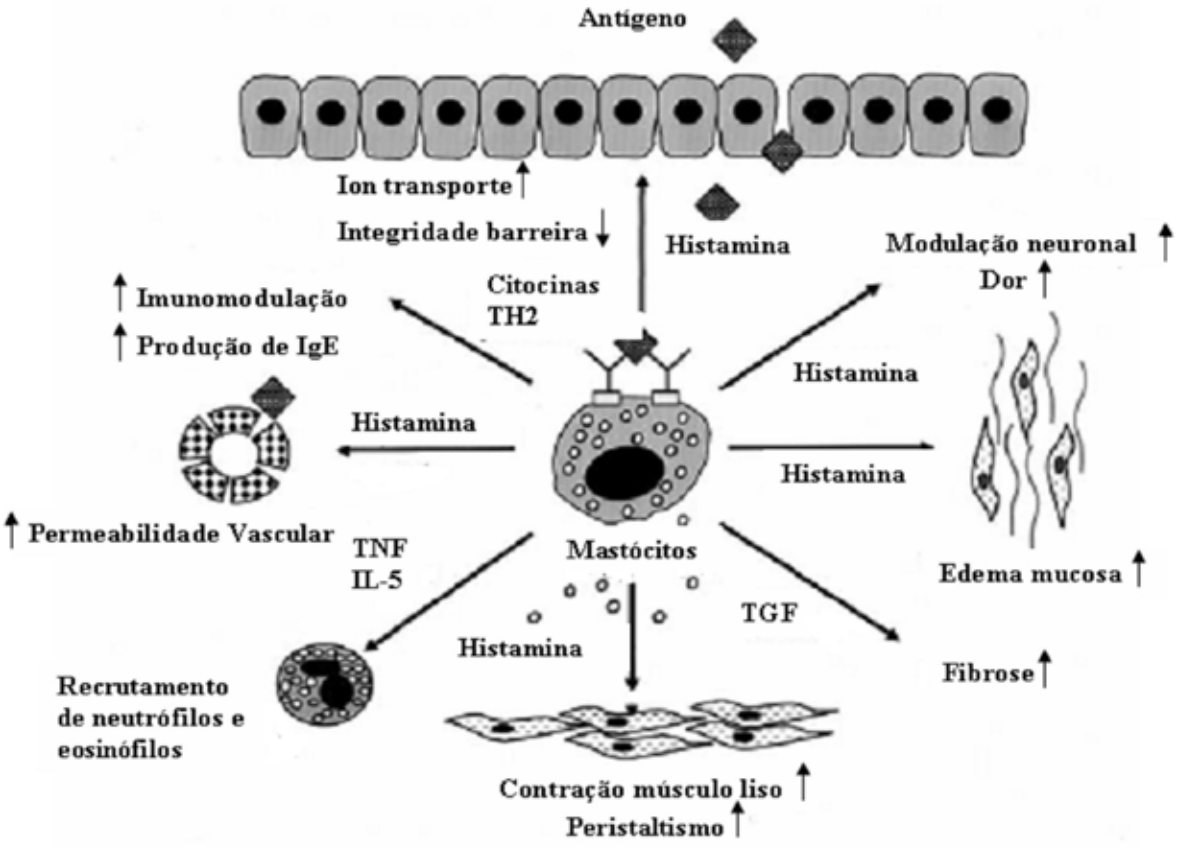
Figure 3: Fatores que afetam o desenvolvimento de reações alérgicas no trato gastrintestinal. Vários fatores ambientais: incluindo bactérias, nutrientes e outros agentes, obtém acesso às células do tecido linfóide associado (TLA), através da barreira epitelial por meio da via transcelular ou paracelular. A tolerância imune a antígenos depende em parte dos genes do hospedeiro que regulam o sistema imunológico, a natureza das células apresentadoras de antígenos que incluem macrófagos, células dendríticas (DC), e células epiteliais; e da produção da citocinas a partir dos linfócitos Th2 (T). A consequência da resposta imune desses vários fatores é a estimulação de linfócitos (B) e ativação de outras células efetoras, incluindo os mastócitos (MC) e eosinófilos (Eo), que resultam na inflamação alérgica. Fonte: adaptado de Bischoff & Crowe, 2005.

2.1.4 Fases da inflamação alérgica

A resposta imune alérgica pode ser dividida em três fases: sensibilização, efetora (constituída de uma fase aguda e de uma fase tardia), e crônica, que pode ser o resultado de repetitivas reações de fase tardia (Bischoff & Crowe, 2005).

A fase de sensibilização é dependente da captação e processamento do antígeno por células apresentadoras, e as subsequentes, apresentação de peptídeos antigênicos a células T $CD4^{+}$. Sob a influência de citocinas específicas, tais como IL-4 e IL-13, células Th0 são transformadas em Th2, as quais, transformam células B em células plasmáticas, que produzem maiores quantidades de IgE específica. Uma vez que basófilos expressam o

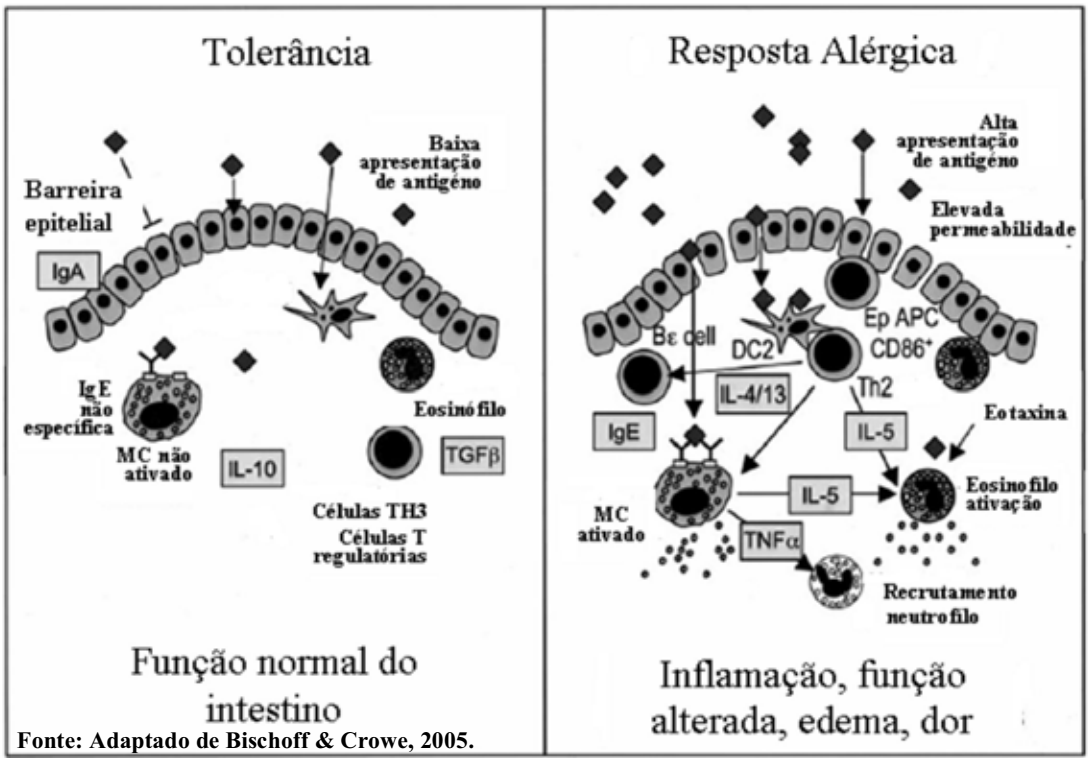
receptor de alta afinidade de IgE, a exposição recorrente ao antígeno pode induzir a uma fase efetora pelo cross-linking de moléculas IgE de superfície. Essa "fase aguda" faz com que haja ativação de mastócitos e basófilos com liberação de histamina, leucotrienos e outros mediadores conhecidos por uma série de efeitos no trato gastrointestinal (**Figura 4**) (Weiner et al., 1994; De Vries et al., 1999; Bischoff et al., 2000; Brandtzaeg, 2002).



Fonte: Adaptado de Bischoff & Crowe, 2005.

Figura 4. Função de células efetoras (mastócitos) durante resposta inflamatória alérgica.

Reações agudas que ocorrem dentro de segundos a minutos podem ser seguidas por uma "reação de fase tardia", que começa dentro 24 horas após a provocação alergênica e é caracterizada por uma infiltração celular do tecido com granulócitos (basófilos, eosinófilos) e linfócitos (principalmente Th2). Todo o processo de manutenção da tolerância e inflamação alérgica é destacado na **Figura 5**.



Fonte: Adaptado de Bischoff & Crowe, 2005.

Figura 5. Mecanismos que conduzem à tolerância e à resposta alérgica no trato gastrintestinal. Na configuração normal (painel esquerdo), pouco antígeno (Ag), a barreira epitelial intacta, produção de IgA, e outros mecanismos inatos de defesa imunológica. Neste cenário, células Th3 predominam com secreção de IL-10 e TGF-β. IgE específica não é produzida, e os eosinófilos e mastócitos (MC) permanecem em um estado de repouso. Em contraste, a permeabilidade epitelial alterada (painel direito) leva a um aumento da carga de antígenos e certas formas de células apresentadoras de antígenos (APC), incluindo células epiteliais (Ep-CD86) e célula dendríticas (DC2), que resulta em ativação de linfócitos B produtores de IgE (célula B) e uma resposta de células Th2 com produção de IL-4, IL-5, e IL-13. Ativação de mastócitos (MC) que libera diversos fatores que atuam no recrutamento de neutrófilos e ativação de eosinófilos.

2.1.5 Efetores e mediadores da resposta alérgica intestinal

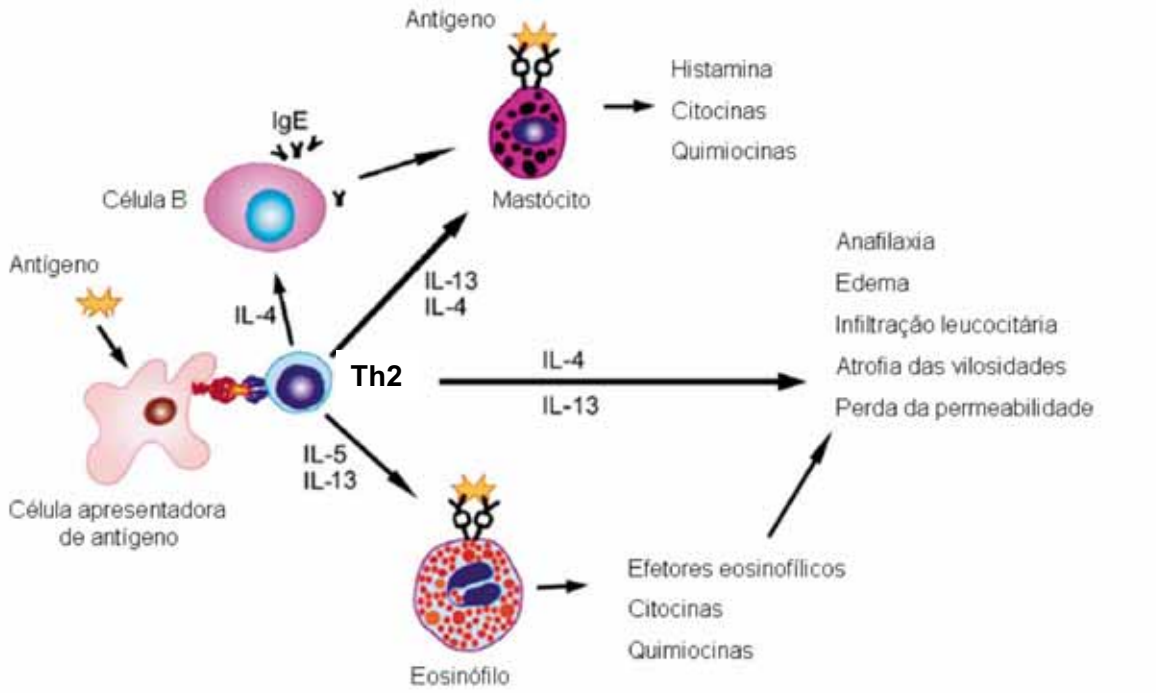
2.1.5.1 Células T $CD4^+$

A manutenção da inflamação alérgica é controlada pela secreção de citocinas produzidas por células T $CD4^+$ antígeno estimulado. As células T $CD4^+$ podem ser divididas em dois subgrupos, com base no seu perfil de citocinas produzidas. As células Th1 produzem interleucina-2 (IL-2), fator de necrose tumoral beta (TNF-β), e interferon gama (IFN-γ), e estão envolvidos na imunidade viral. As células Th2 secretam interleucina-4 (IL-4), interleucina-5 (IL-5), interleucina-10 (IL-10) e interleucina-13 (IL-13), e promovem as respostas de anticorpos e inflamação alérgica. IL-4 e IL-13 são fatores críticos para a regulação das células Th2, pois afetam diretamente a produção de IgE por linfócitos B, o funcionamento dos mastócitos, e a modulação da produção de quimiocinas. Em conjunto com outras citocinas do tipo Th2, bem como IL-1β e TNF-β, IL-4, também podem regular o tráfico, ativando sistemas de adesão ao endotélio vascular. IL-5 regula o crescimento, diferenciação, ativação e sobrevida dos eosinófilos e fornece sinais essenciais para a amplificação da eosinopoese e para o recrutamento destes para locais de inflamação alérgica (Eigenmann, 2002; Turcanu et al., 2003).

2.1.5.2 Mastócitos e eosinófilos

Mastócitos, eosinófilos e basófilos são reconhecidos não apenas como células efetoras da inflamação alérgica, mas também como células que contribuem para a manutenção da homeostase no intestino (Falcone et al., 2000; Gurish & Austen, 2001; Dombrowicz & Capron, 2001). Os mediadores inflamatórios produzidos por mastócitos e eosinófilos são responsáveis pelos sintomas clínicos e disfunção orgânica que ocorre durante as reações alérgicas. O elevado nível de histamina, proteína catiônica eosinofílica, IL-5, e fator de necrose tumoral (TNF-α) têm sido identificados no soro, urina, lavado do intestino e fezes de indivíduos alérgicos (Bengtsson et al., 1997; Santos et al., 1999; Schwab et al., 2003). Outra evidência da ativação de mastócitos e eosinófilos são estudos histológicos mostrando degranulação, produção de citocinas, e infiltração linfocitária após testes de provocação alergênica (Bischoff et al., 1997). Os mecanismos que regulam o

controle de inflamação e perda de tolerância com subsequente ativação de mastócitos e eosinófilos no curso de hipersensibilidade alimentar são resumidos na **Figura 6**.



Fonte: Adaptado de Foster et al., 2002.

Figura 6. Controle celular e molecular da alergia alimentar. As células Th2 desempenham um papel central na orquestração da inflamação alérgica que resulta no distúrbio de tolerância alimentar, como anafilaxia, edema, infiltração leucocitárias, atrofia e perda de permeabilidade. Após a ativação por antígeno, as células Th2 produzem IL-4 necessário para a maturação das células B e síntese de IgE; IL-13 e IL-5 para o crescimento e diferenciação de eosinófilos; IL-4 e IL-13 para o desenvolvimento dos mastócitos.

Mastócitos humanos produzem TNF-α causando recrutamento de neutrófilos; e IL-5, que promove mobilização de eosinófilos. IL-4 não é produzido pelos mastócitos em condições normais no ser humano, mas age como um regulador central na produção de outras citocinas pelos mastócitos (Lorentz et al., 2000).

2.1.5.3 Citocinas

Citocinas são proteínas produzidas durante a fase de ativação e fase efetora da imunidade para mediar e regular a resposta inflamatória e imunitária.. Estas só estimulam as células com receptores específicos na membrana da célula alvo. Apresentam uma vida média curta e são moléculas pleiotrópicas (Cavaillon, 2005). O paradigma observado em alergias alimentares implica fortemente as citocinas Th2 (Wills-Karp, 1999). Essas citocinas promovem a diferenciação, sobrevivência e função de células efetoras, e agem diretamente sobre células do parênquima para provocar o estado alérgico.

2.1.5.3.1 IL-4

É uma citocina que induz diferenciação de linfócitos Th0 em Th2, e após essa ativação, as células Th2 subseqüentemente produzem mais IL-4. Embora várias células efetoras possam contribuir para alergia, grande parte dos sistemas de suporte experimental dá um papel central para a célula Th2, que pode participar diretamente ou indiretamente da coordenação da atividade de outras células. IL-4, além de ser um produto das células Th2, atua como uma citocina autócrina para o crescimento e desenvolvimento dessas (Sokol et al., 2008). Apesar de exposição intensa aos alérgenos, na ausência de IL-4, células Th2 não desenvolvem ou não podem ser sustentadas *in vivo*. Assim, IL-4 é a principal citocina na inflamação alérgica ao regular o crescimento e funcionamento de células B e T, imunoglobulinas e células hematopoiéticas (Paul, 1991).

2.1.5.3.2 IL-5

É uma proteína produzida pela subpopulação de células Th2, bem como por mastócitos ativados. A principal função da IL-5 é estimular o crescimento e a diferenciação dos eosinófilos e ativar eosinófilos maduros. Em vez de coordenar o desenvolvimento de células T efetoras ou secreção de anticorpos por células B, os efeitos da IL-5 são confinados, principalmente, à sobrevivência, ao crescimento e à função de eosinófilos (Sanderson, 1992). A contribuição da IL-5 para doença alérgica foi muito facilitada pela geração de camundongos deficientes no gene IL-5. Em contraste a camundongo normal, IL-

5-deficientes não gera eosinofilia na medula óssea, sangue ou pulmão em resposta à provocação de alérgenos respiratórios (Foster et al., 1996).

2.1.5.4 Quimiocinas

Quimiocinas (citocinas quimiotáticas) é uma família de pequenas moléculas que está, principalmente, envolvida na regulação do tráfico leucocitário durante a homeostase e também durante a inflamação (Moser & Loetscher, 2001). A família de quimiocinas foi subdividida em quatro grupos (C, CC, CXC, e CX3C), com base no espaçamento de conservados grupos de cisteínas. Geralmente, quimiocinas CXC regulam o tráfico de neutrófilos e linfócitos, enquanto quimiocinas CC modulam o tráfico de um número variado de células, incluindo basófilos, mastócitos, eosinófilos, células dendríticas e macrófagos. Quimiocinas interagem quase que exclusivamente, com leucócitos e tem a capacidade de modular seletivamente esse tráfico, incluindo a expressão e ativação de receptores (Moser & Loetscher, 2001).

2.1.5.4.1 RANTES

RANTES (Regulador da secreção e expressão de células T após ativação) pertence à família CC, é uma citocina quimiotática produzida por células T, células epiteliais, fibroblastos, células endoteliais, e eosinófilos (Schall et al., 1988; Devergne et al., 1994; Stellato et al., 1995). RANTES induz migração dirigida de células T $CD4^+$ e monócitos (Schall et al., 1990), quimiotaxia e ativação de eosinófilos (Kuna et al., 1995), e migração de eosinófilos transendotelial (Ebisawa et al., 1994). RANTES também ativa basófilos e induz a liberação de histamina (Kuna et al., 1992).

2.1.5.4.2 Eotaxina

Eotaxina pertence à família CC de quimiocinas e tem cerca de 50% de homologia sequencial com as proteínas quimiotáticas de monócitos (MCP), alguns dos quais são conhecido por ser potente indutor do rápido recrutamento de eosinófilos (Baggiolini et al., 1997; Rothenberg, 1999). Investigações em cobaias sugerem que a eotaxina e IL-5 agem

cooperativamente para promover o recrutamento de eosinófilos (**Figura 7**) do sangue para os tecidos (Mould et al., 1997).

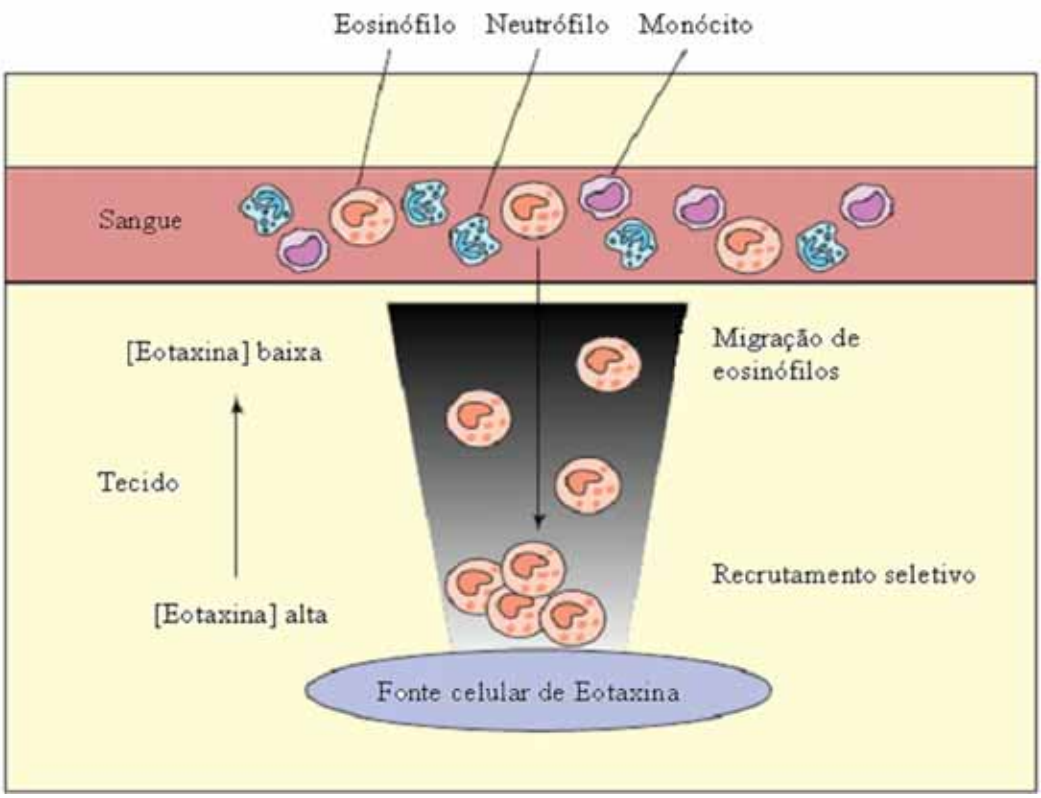


Figura 7. Eotaxina estimula o recrutamento seletivo de eosinófilos do sangue para os tecidos. Quimiotaxia é a migração dos leucócitos na direção do aumento da concentração do quimiotático. Eotaxina é um potente fator para eosinófilos, mas não estimulam a quimiotaxia de neutrófilos ou monócitos (Fonte: Adaptado de Rankin et al., 2000).

A eotaxina, além de estimular a quimiotaxia de eosinófilos, induz sua agregação (Elsner et al., 1996). Eotaxina também regula a subunidade CD11b da integrina (Tenscher et al., 1996) e, assim, proporciona a adesão dos eosinófilos às células endoteliais (Burke-Gaffney & Hellewell, 1996). Em sinergismo com IL-5, a eotaxina pode estimular a rápida liberação de eosinófilos e seus progenitores da medula óssea (Palframan et al., 1998), resultando em uma rápida eosinofilia sanguínea (**Figura 8**).

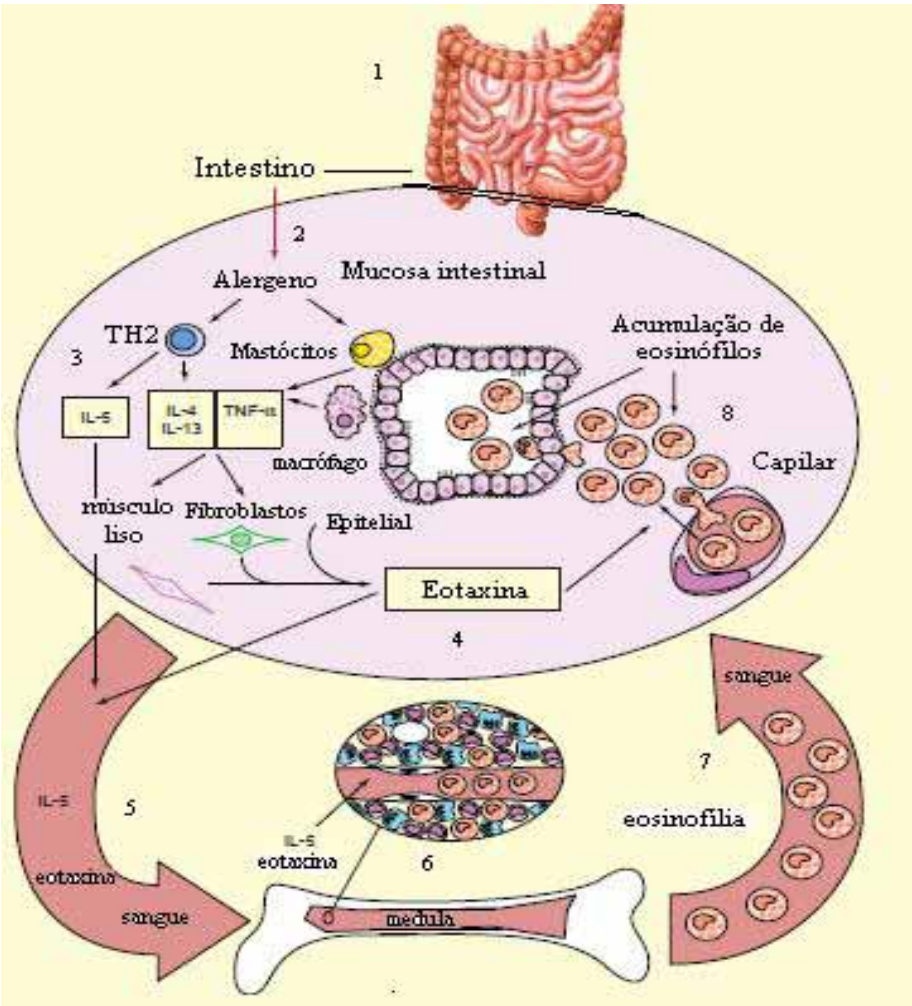


Figura 8. O papel da eotaxina na patogênese da inflamação alérgica. Alérgenos (1) ativam mastócitos e células Th2 na mucosa (2) do intestino. A ativação de células Th2 produz as citocinas: IL-5, IL-4 e IL-13 (3), enquanto os mastócitos e macrófagos geram TNF-α (3). IL-4, IL-13 e TNF-α agem sinergicamente para estimular a geração de eotaxina por células epiteliais e células musculares lisas e fibroblastos (4). IL-5 e eotaxina gerada na mucosa seguem para a circulação (5). IL-5 e eotaxina agem sinergicamente para mobilizar os eosinófilos da medula óssea (6). A rápida liberação de eosinófilos da medula gera uma eosinofilia (7). Finalmente, os eosinófilos circulantes são recrutados do sangue para o tecido intestinal por eotaxina (8). Fonte: Adaptado de Rankin et al., 2000.

2.1.5.4.3 Regulação da produção de quimiocinas por citocinas

Entre as citocinas com ampla atividade pró-inflamatória, TNF-α e IL-1 têm demonstrado ser potentes indutores de quimiocinas em uma variedade de células *in vitro* (Rathanaswami et al., 1993). IFN-γ potencializa enormemente a produção de RANTES (Stellato et al., 1995) e menos marcadamente de eotaxina, que é induzida em células epiteliais por TNF-α (Lilly et al., 1997; Matsukura et al., 2003). Duas das citocinas derivadas de células Th2, IL-4 e IL-13, induzem a expressão da eotaxina (Rothenberg et al., 1995; Mochizuki et al., 1997). Assim, IL-4 e IL-13 podem levar ao recrutamento seletivo de eosinófilos por indução de VCAM-1 (molécula de adesão celular ao endotélio vascular) e liberação de eotaxina (Schleimer et al., 1992).

2.1.6 Classificação das alergias alimentares

Uma abordagem da fisiopatologia das alergias alimentares em crianças e adultos mostra que os mecanismos de sensibilização alérgica ocorrem por meio da formação de anticorpos IgE ou da resposta imune aumentada da célula T sem formação de anticorpos ou, ainda, de mecanismo misto (Sampson, 2004), como mostrado na **Tabela I**.

Tabela I. Desordens de alergia alimentar

IgE mediada	
Gastrintestinal	Síndrome de alergia oral, anafilaxia gastrintestinal
Generalizado	Choque anafilático
Conjunto IgE e não IgE (celular) mediadas	
Gastrintestinal	Esofagite e gastrenterites eosinofílicas alérgicas
Não IgE (celular) mediada	
Gastrintestinal	Proteína alimentar – enterocolite, enteropatia e doença celíaca

Fonte: Adaptado de Sampson, 2004.

2.1.7 Patogênese das alergias alimentares

2.1.7.1 Alergias alimentares mediadas por IgE

Reações mediadas por IgE, que constituem a maioria das reações alérgicas aos alimentos, têm sido as mais bem estudadas. Se a tolerância oral não é desenvolvida, existe a produção de IgE específica para tais alimentos. IgE estão ligadas a receptores específicos de alta afinidade presentes nos mastócitos, células presentes na mucosa e pele, e basófilos que circulam no sangue. Essas células têm numerosos grânulos citoplasmáticos em que mediadores pré-formados são armazenados, incluindo, principalmente, a histamina (Sampson, 2004). Quando as proteínas alergênicas são absorvidas no intestino, entram em contato com IgEs específicas ligadas aos mastócitos/basófilos, o que desencadeia um estímulo para estas células, que desgranulam, lançando mediadores no microambiente em torno e sintetizando novos mediadores (prostaglandinas, leucotrienos, e citocinas).

Uma reação imediata segue alguns minutos após o contato com o alérgeno, devido, à histamina. Na base da reação, há vasodilatação, exsudação de líquidos dos tecidos, contração do músculo liso e secreção de muco. A resposta de fase tardia segue a reação imediata, que começa 4-6 horas após o contato com o alérgeno e continua por vários dias. Esta resposta é causada por mediadores quimiotáticos liberados, que promovem recrutamento seletivo de células inflamatórias, principalmente eosinófilos e neutrófilos, os quais infiltram o tecido produzindo uma inflamação que se prolonga por alguns dias. Os dois elementos clínicos necessários para apoiar uma alergia alimentar IgE mediada é a presença de anticorpos IgE específicos e uma relação comprovada entre a ingestão do alimento e o aparecimento dos sintomas (Ortolani & Pastorello, 2006).

2.1.7.2 Alergias alimentares mediadas por não-IgE (celular)

Em geral, durante os primeiros anos de vida, as alergias mediadas por células são mais freqüentes, quando comparada com as medidas por IgE (potencialmente fatais), devido à imaturidade do sistema imune da mucosa intestinal. No entanto, este tipo particular não pode ser bem rastreado devido à falta de uma relação causal facilmente apreciável entre alérgenos e características clínicas. Os sintomas podem ocorrer horas mais

tarde, tornando difícil o diagnóstico clínico, mesmo que a presença dessas alergias ter sido descrita na literatura há mais de 60 anos (Jyonouchi, 2008).

Em oposição às alergias alimentares mediadas por IgE, note-se que a maioria dos pacientes revela teste cutâneo com reatividade negativa e ausência de alérgenos IgE específica. Devido à má absorção crônica, a atrofia das vilosidades pode ser encontrada, mas os achados histológicos variam consideravelmente na literatura, principalmente descrevendo alterações inflamatórias não específicas (Sicherer, 2005; Sicherer & Sampson, 2006). A reatividade imune deste tipo de alergia foi inicialmente abordada, explorando a presença de anticorpos IgG ou IgA e também a proliferação de células T. No entanto, esses parâmetros mostraram ser de baixo valor clínico e estudos recentes estão focados sobre o papel das células T específicas e a produção de citocinas e quimiocinas (Jyonouchi, 2008). Enterocolite, enteropatia e doença celíaca são desordens gastrintestinais eosinofílicas, mas só parece envolver um mecanismo não IgE mediada. As lesões estão confinadas ao intestino e consistem de edema da mucosa, com infiltração linfocítica e de eosinófilos no epitélio e na lâmina própria (Snyder et al., 1987).

2.1.8 Modelo animal para alergia alimentar

Os modelos animais, apesar de suas limitações individuais, são ferramentas úteis para o estudo de reações alérgicas *in vivo* (Helm, 2002). Eles têm sido fundamentais no avanço da nossa compreensão dos mecanismos fisiopatológicos, ao avaliar a alergenicidade de produtos alimentares e o sucesso de novas terapêuticas (Kitagawa et al., 1995; Niggemann et al., 2001; Knippels & Penninks, 2002). Animais oferecem a capacidade de estudos de sensibilização, estudos que não são possíveis em seres humanos por razões éticas óbvias. Modelos variam em termos de animais utilizados (camundongo, rato, porco, cachorro), protocolos de sensibilização (tipo de alérgenos alimentares, dose, via de administração, uso de adjuvantes), e os métodos utilizados para avaliar a resposta alérgica (quantificação de mediadores da reação inflamatória, os ensaios funcionais da função intestinal, e estudos morfológicos). Também é importante considerar o background genético do animal na busca por uma resposta imune adequada. Por exemplo, ratos Brown Norway e Camundongos Balb/c são bons produtores de IgE e têm sido utilizados no estabelecimento de modelos de alergia alimentar (Fritsche, 2003).

Apesar dos benefícios de modelo animal de alergia alimentar, há inexistência de um modelo animal que possa identificar alérgenos alimentares conhecidos, predizer o potencial alérgico de alimentos proteicos, ou imitar a sensibilização alérgica alimentar e reações em humanos é uma importante limitação (Helm, 2002).

2.2 Alérgenos alimentares

Os principais alérgenos alimentares são geralmente proteínas relativamente estáveis ao calor, à acidez e a degradação proteolítica. No entanto, é claro que aspectos adicionais, tais como preparação do alimento, pode elevar sua antigenicidade potencial. Um exemplo é a teoria proposta para explicar uma maior taxa de alergia ao amendoim em países ocidentalizados, onde o amendoim é consumido torrado, em comparação com menor taxa de prevalência na China, onde é cozido ou frito (Sicherer & Sampson, 2007). Em outro exemplo clássico, estima-se que 70-80% de crianças alérgicas a ovos cozidos podem tolerar formas da proteína desnaturada, mas não na forma nativa (Beyer et al., 2001; Lemon-Mule et al., 2008).

2.2.1 Classificação

Os alimentos vegetais mais antigênicos pertencem a poucas famílias de proteína. Muitos alérgenos pertencem à superfamília das cupinas (agrupa as proteínas tipo germinas, leguminas, vicilinas, proteínas de armazenamento de sementes 7S e 11S) ou à superfamília das prolaminas (albuminas 2S, inibidores de α-amilase/tripsina, prolaminas e proteínas de armazenamento de cereais). As proteínas relacionadas à patogênese (PRs) representam um grupo heterogêneo de 14 famílias de proteínas vegetais que estão envolvidas na resistência de plantas a patógenos ou a condições adversas ambientais. Neste grupo, também estão incluídas lectinas vegetais (Breiteneder & Ebner, 2000).

Proteínas de armazenamento é a causa da bem conhecida reação alérgica a amendoim e cereais. PRs são também responsáveis pela reação alérgica ao pólen (Breiteneder & Ebner, 2000). Todas as classes de alérgenos, sua classificação e fonte estão sumerizadas na **Tabela II**.

Tabela II – Principais alérgenos alimentares protéicos vegetais.

Família	Classificação da proteína	Alérgeno / fonte do alérgeno
Superfamília das cupinas		
Vicilinas	Proteína de armazenamento das sementes	Ara h 1 (amendoim), Jug r 2 (noz)
Leguminas	Proteína de armazenamento das sementes	Ara h 3/4 (amendoim), Cor a 9 (avelã)
Superfamília das prolaminas		
Albuminas 2S	Proteína de armazenamento das sementes	Ber e 1 (castanha do Brasil), Ses i 2 (gergelim)
Cereal inibidores de α-amilase/tripsina	Inibidores de protease e α-amilase	Inibidor da alfa-amilase dimérica do arroz
Prolaminas	Proteína de armazenamento das sementes (cereais)	Tri a 19 (trigo), Sec c 20 (centeio)
Proteínas relacionadas à patogênese (PRs)		
Proteínas do tipo PR-2	β-1,3-glucanases	Frutas, legumes
Proteínas do tipo PR-3	Proteínas básica I quitinases	Pers a 1 (abacate), castanha, banana
Proteínas do tipo PR-4	Quitinases	Nabo, sabugueiro
Proteínas do tipo PR-5	Proteína com domínio taumatina	Mal d 2 (maçã), P23 (pimentão)
Proteínas do tipo PR-10	Proteínas homólogas a Bet v 1	Pru av 1 (cereja), Pru ar 1 (damasco), Pyr c 1 (pêra), Dau c 1 (cenoura), PCPR (salsa), pSTH (batata)
Proteínas do tipo PR-14	Proteína transferência de lipídeos	Mal d 3 (maçã), Gly m 1 (soja)
Profilinas	Proteína ligante de actina	Ara h 5 (Amendoim), Gly m 3 (soja), Pyr c 4, (pêra)
Lectinas	Proteína ligante de gliconjugados	Aglutinina (Amendoim), WGA (trigo)

Fonte: Adaptado de Breiteneder & Ebner (2000)

Como mostrado na **Tabela II**, os principais alérgenos alimentares vegetais pertencem a restritas famílias de proteínas com estruturas conservadas e atividades biológicas que desempenham um papel na promoção de propriedades alergênicas. Além disso, está se tornando evidente que o nível de exposição e as propriedades moleculares de um alérgeno têm um papel importante na determinação do potencial alergênico, embora a base molecular para estes efeitos ainda não seja compreendida. De uma maneira geral, a alergenicidade de uma proteína alimentar é determinada por uma soma de fatores, incluindo, sua abundância, e a estabilidade frente ao processamento e a digestão (Breiteneder & Mills, 2005b).

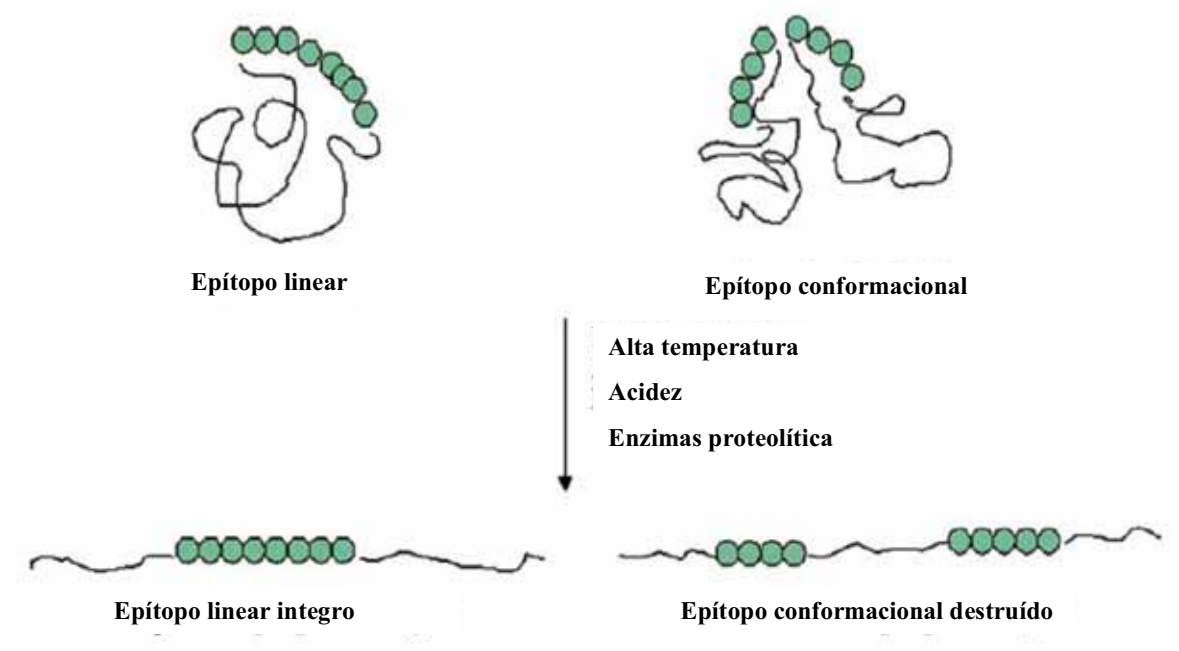
2.2.2 Estrutura molecular dos alérgenos alimentares

O banco de dados, Pfam, de família de proteínas (Bateman et al., 2004) é uma grande coleção de famílias e domínios proteicos. Ele classifica sequências de proteína vegetal em famílias com base na homologia, que está relacionado com a conservada estrutura tridimensional e, possivelmente, com a função biológica intrínseca. Através deste importante banco de dados, foi possível caracterizar quase todos os alérgenos alimentares

vegetais e dividi-los em três grupos estruturalmente homólogos (Breiteneder & Radauer, 2004). A superfamília das prolaminas é o principal grupo de alérgenos alimentares vegetais (Breiteneder e Radauer, 2004, Mills et al., 2004). Todos eles apresentam baixa massa molecular, são abundantes em cisteína e têm a mesma estrutura tridimensional, ricos em alfa-hélices, sendo estáveis ao processamento térmico e à proteólise. As cupinas pertencem a uma superfamília funcionalmente diversa de proteínas que compartilham um domínio beta-barril estrutural e compreende as principais globulinas de armazenamento de leguminosas e nozes. Também exibem estabilidade ao processamento térmico e à proteólise intestinal. Enquanto isso, alérgenos do tipo Bet v 1 são bastante instáveis ao aquecimento e digestão. Consequentemente, sintomas e efeitos alérgicos são mais restritos à cavidade oral.

2.2.3 Epítopos lineares e estruturais dos alérgenos

Uma resposta IgE específica ou celular reconhece estruturas moleculares conformacionais e lineares em proteínas alergênicas. Um epítopo é linear quando a IgE liga-se a uma série de aminoácidos adjacentes, sem exigência de uma estrutura secundária ou terciária, enquanto isso, um epítopo conformacional é estritamente dependente do enovelamento da cadeia proteica. Epítopos lineares mantém sua capacidade de ligação, mesmo após a modificação estrutural causada por fortes condições ácidas do estômago ou a atividade proteolítica das enzimas gastrintestinal (**Figura 9**). Assim, como no processo digestivo, epítopos conformacionais e lineares são diferentemente afetados pelo processamento de alimentos (Restani et al., 2004; Nowak-Wegrzyn & Sampson, 2004).



Fonte: Nowak-Wegrzyn & Sampson, 2004

Figura 9. Epítopo linear e conformacional de alérgenos alimentares.

2.2.4 Relação entre alergenicidade e as características moleculares dos alérgenos

2.2.4.1 Abundância em alimentos

As sementes e nozes contêm proteínas de armazenamento que podem ser responsáveis por 50% ou mais das proteínas totais do alimento. Alérgenos alimentares que provocam reações de hipersensibilidade por meio do trato gastrintestinal estão presentes em, pelo menos, 1% do teor de proteína total de alimentos de origem vegetal. No entanto, algumas proteínas que estão presentes em todas as plantas em quantidades muito grandes, como a enzima ribulose-1,5-bisfosfato carboxilase (Rubisco) é responsável por 30-40% de proteína de folhas ─ nunca foram relatadas como alérgenos. Assim, a quantidade de proteína por si só não explica a sua alergenicidade. Enquanto a abundância é um fator importante, é provavelmente secundária quando comparada à estabilidade estrutural da proteína alergênica (Breiteneder & Mills, 2005b).

2.2.4.2 Estabilidade frente ao processamento de alimentos e digestão

A estrutura tridimensional compacta, pontes de dissulfeto, e glicosilação contribuem fortemente para a estabilidade da proteína (Breiteneder e Mills, 2005a). Esses fatores são relevantes tanto para a resistência das proteínas à desnaturação pelo processamento do alimento como pelas condições adversas do trato gastrintestinal. Algumas proteínas formam uma cavidade, enquanto outras possuem um sítio de ligação em que ligantes se encaixam. Quando o canal é ocupado por ácidos graxos ou moléculas de fosfolipídios; nsLTPs (proteínas não-específicas de transferência de lipídeos), que possuem um bolso de lipídios vinculativo, mostram uma maior estabilidade.

Uma das estruturas características claramente relacionadas com a estabilidade é a presença de pontes de dissulfeto. Pontes dissulfeto inter e intracadeias restringem a perturbação estrutural por calor ou químicos e, freqüentemente, qualquer modificação é reversível. Importantes alérgenos vegetais têm um elevado número de pontes de dissulfeto e incluem membros da superfamília das prolaminas (nsLTPs, albuminas 2S, inibidores α-amilase/tripsina), bem como de proteínas relacionadas à patogênese (PRs). A glicosilação pode ter um efeito significativo na estabilização da estrutura de proteínas (Breiteneder e Mills, 2005b). Finalmente, as mudanças estruturais induzidas pelo processamento de alimento, tais como tratamento térmico, podem alterar o transporte intestinal de alguns alérgenos, como mostrado para a β-lactoglobulina desnaturada (Rytkönen et al., 2006).

2.2.4.3 Interação e agregação

Muitos alérgenos alimentares vegetais são capazes de se associar com as membranas celulares ou com outros tipos de lipídios encontrados em alimentos ou mostrar uma propensão a se agregar como resultado do processamento alimentar (Breiteneder e Mills, 2005a). A propensão de certas proteínas para agregar pode afetar sua capacidade de sensibilização geralmente por aumentar a sua imunogenicidade. Um exemplo são as termoestáveis globulinas 7S e 11S de soja, onde, ao que parece, o domínio beta-barril estrutural permanece intacto, mas o desdobramento de outras regiões da proteína resulta em uma perda de estrutura, levando à formação dos grandes agregados (Breiteneder e Mills, 2005b). Amendoins, por exemplo, são, muitas vezes, submetidos a processamento térmico

em baixos níveis de água, como a torrefacção. Assim, proteínas do amendoim tornam-se mais termoestáveis em sistemas de baixa água, ao mesmo tempo em que reações de glicação em moléculas individuais aumentam a sua antigenicidade. Portanto, a interação com lipídios e a formação de agregados pode contribuir para a alergenicidade de proteínas vegetais em conjunto com a quantidade de proteína ingerida e da estabilidade ao processamento e à digestão (Breiteneder e Mills, 2005b).

2.2.5 Lectinas

Lectinas, também conhecidas como fitoaglutininas, é uma classe de proteínas que se ligam seletivamente e de forma reversível a carboidratos. Lectinas são encontradas em sementes, especialmente as de legumes. Algumas lectinas reagem de forma não específica com IgE, induzem liberação de histamina, e podem, assim, induzir alergia (Shibasaki et al., 1992). A aglutinina de amendoim tem sido identificada como uma lectina que é expressamente reconhecida por IgE em pacientes alérgicos a amendoim (Burks et al., 1994). Estudos em animais mostraram que as lectinas têm uma ampla gama de efeitos que podem ser relevantes para patologias digestivas humanas. Estes incluem alterações na diferenciação, bem como na proliferação de células intestinais e do cólon. Lectinas alimentares podem também afetar a flora intestinal e ser um importante fator para o agravamento de doenças associadas (Hamid & Masood, 2009).

2.2.5.1 Lectina da semente da leguminosa *Cratylia mollis* (Cramoll)

Cramoll é uma lectina isolada das sementes de *Cratylia mollis* (Correia e Coelho, 1995) da família Leguminosae. Cramoll tem especifica ligação para glicose / manose (De Souza et al., 2003). Cramoll é composta por três fragmentos moleculares, P1, P2 e P3, com massa molecular de 30.000 Da (proteína intacta - P3), 16.000 Da (P1) e 14.000 Da (P2).

A determinação da sequência N-terminal dos componentes mais leves indicou que (P1) é a porção N-terminal e (P2) é a porção C-terminal da proteína intacta (P3). Sua estrutura secundária nativa é rica em folha e volta beta (Varejão et al., 2010). Apesar de não ser uma glicoproteína e não ter pontes dissulfeto, Cramoll é termoestável e resistente a proteases (**Figura 10**).

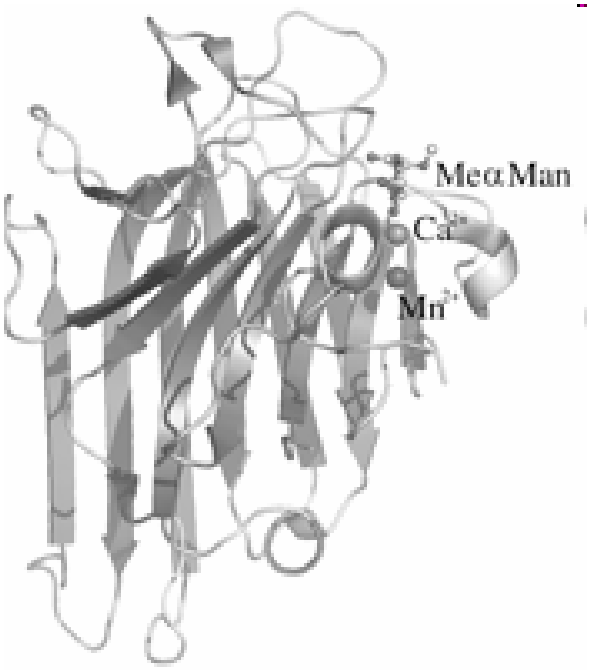


Figura 10. Estrutura tridimensional da Cramoll. Três tipos de β-folhas são observados: seis folhas β relativamente planas; sete curvas presas de folhas β dianteiras que são fechadas na parte traseira com folhas α, e cinco pequenas folhas β superior que desempenha um importante papel na sustentação das grandes folhas β. Quatro alças associadas com a face côncava na forma de folhas β estão à frente de uma depressão rasa, onde se localiza o sitio de ligação ao carboidrato e os dois sítios metálicos. (Fonte: De Souza et al., 2003).

Cramoll exibe toxicidade para linfócitos e induz uma atividade imunomoduladora por meio da produção de IFN-γ, IL-2, IL-6 e óxido nítrico (De Melo et al., 2010). Além disso, Cramoll aumentou a produção intracelular de espécies reativas de oxigênio (ROS), dos níveis de Ca^{2+}, e da expressão de interleucina (IL)-1beta (De Melo et al., 2011). As implicações nutricionais da Cramoll foram avaliadas e revelaram atraso no desenvolvimento de ratos devido à redução na digestibilidade protéica e toxicidade hepática (Oliveira, 2002).

2.2.5.2 Lectina do feijão da leguminosa *Canavalia ensiformis* (Con-A)

Concanavalina A (Con-A) é uma lectina originalmente extraída do feijão *Canavalia ensiformis*. Con-A liga-se especificamente a grupos α-D-manosil e α-D-glicosil. Estudos têm confirmado que Con-A existe sobre duas formas, uma subunidade intacta e outra subunidade composta por dois fragmentos, Pl (13.000 Da) P2 (11.000 Da) (Edelman et al.,

1972). O elemento estrutural predominante na cadeia polipeptídica são duas folhas betas antiparalelas (Edelman et al., 1972). Con-A exibe um padrão de oligomerização e exibe a mesma resistência proteolítica observada na Cramoll (**Figura 11**).

Con-A é conhecida por sua capacidade de estimular linfócitos (Dwyer & Johnson, 1981) e induzir basófilos humanos a secretar IL-4 e IL-13, os promotores chave para respostas Th2 e para a síntese de IgE (Haas et al., 1999). Ela também promove a secreção de TNF-α e IFN-γ em modelos de injúria hepática (Ksontini et al., 1998; Tsai et al., 2011).

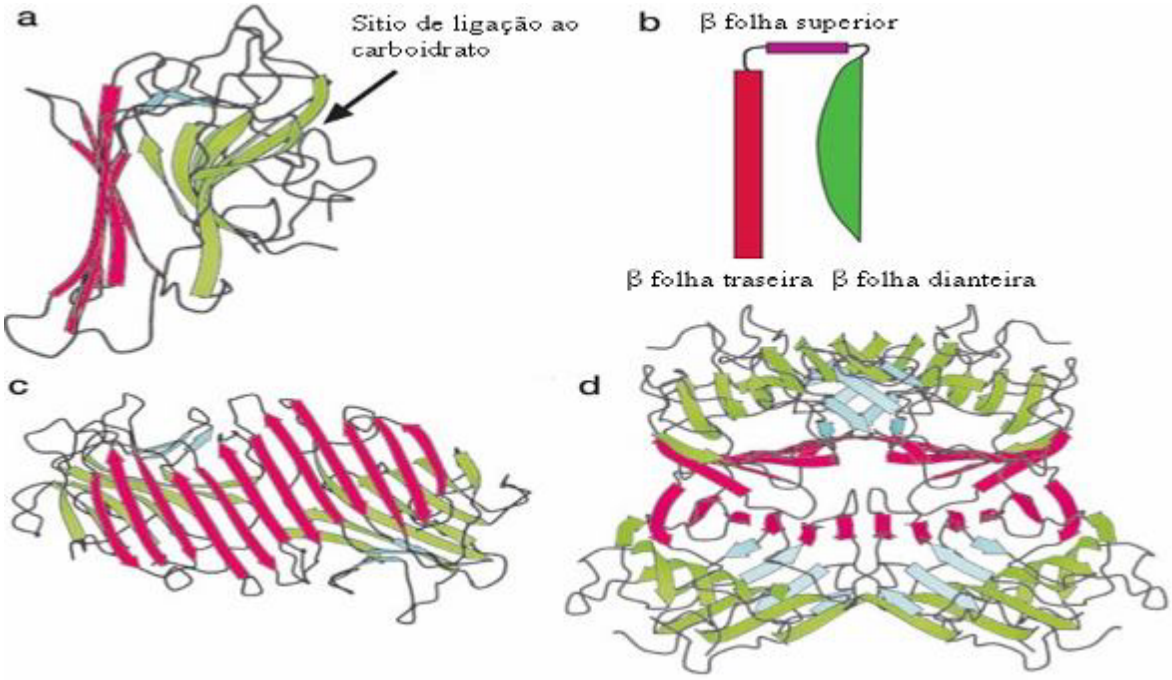


Figura 11. Estrutura de Concanavalina A (Con-A). (a) A estrutura terciária do monômero é mais bem descrito como um "fold jelly-roll." (b) Essa dobra consiste de seis folhas β relativamente planas (vermelho); sete curvas presas de folhas β dianteiras (verde), que são fechadas na parte traseira com folhas α, e cinco pequenas folhas β superior (rosa). (c) Dimerização de Con-A envolve o alinhamento antiparalelo lado a lado das folhas β traseiras. (d) A tetramerização de Con-A ocorre por uma associação da folhas β traseiras dos dois dímeros. (Fonte: Adaptado de Srinivas et al., 2001).

2.2.5.3 Aglutinina de gérmen de trigo (WGA)

WGA é uma proteína homodimérica composta de duas subunidades de 17 kDa contendo 16 pontes dissulfeto. Os monômeros associam-se formando um duplo glóbulo simétrico (**Figura 12**).

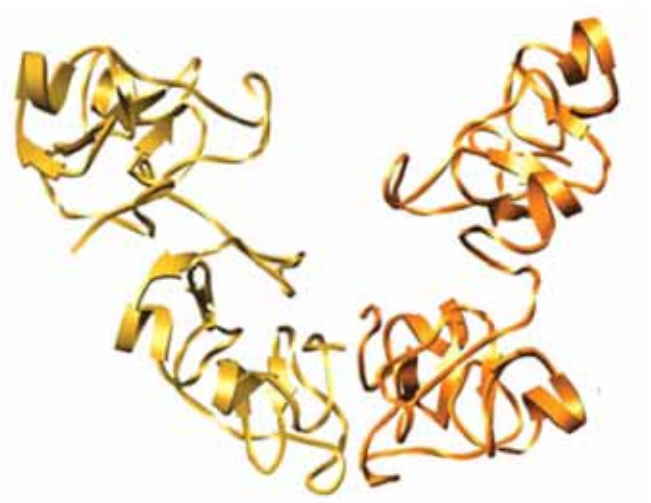


Figura 12. Representação esquemática da estrutura tridimensional do dímero WGA. (Fonte: DeMarco & Woods, 2008).

WGA é estável ao calor, resistente à degradação proteolítica e produz perdas de nitrogênio endógeno e deprimidas taxa de crescimento em animais jovens sensibilizados (Cordain, 1999). Em ratos, WGA se liga a glicanos na superfície de células epiteliais na base das vilosidades intestinais. O resultado dessas mudanças estruturais inclui o aumento da permeabilidade intestinal para diferentes marcadores de permeabilidade (Watzl et al., 2001). WGA interage com o receptor de IL-2 em Linfócitos T e B, e, assim, prejudica a função de linfócitos *in-vitro* (Watzl et al., 2001). WGA se liga à imunoglobulina (Ig) em leucócitos de pacientes alérgicos e induz a secreção de histamina, bem como a secreção de IL-4 e IL-13 por basófilos humanos (Haas et al., 1999). A produção de TNF-α, IL-1β, IL-12 e IFN-γ macrófagos *in vitro* foi também observado (Sodhi & Kesherwani, 2007).

2.3 O processamento de alimento

2.3.1 A deterioração de alimentos e fatores biológicos

Alimentos naturais são compostos, principalmente, de carboidratos, proteínas e gorduras. Sob condições armazenamento natural, os alimentos começam a deteriorar-se. Quando o tecido é danificado, a deterioração começa com a secreção de proteases internas (como quimotripsina e tripsina), lisozimas e lipases que hidrolisam proteínas, amido e gorduras. A exposição de alimentos e células danificadas ao meio ambiente atrai

microorganismos (por exemplo, bactérias, fungos e vírus) e insetos, que, por sua vez, aceleram ainda mais a decomposição dos alimentos (Smith & Hui, 2004).

2.3.2 A deterioração de alimentos e fatores químicos e físicos

Em muitos casos, quando os alimentos são oxidados, eles se tornam menos desejáveis ou, até mesmo, rejeitado. O odor, sabor e cor podem mudar, e alguns nutrientes podem ser destruídos. Danos oxidativos resultam na libertação de odores durante o colapso de ácidos graxos insaturados. Esses produtos incluem aldeídos, cetonas e ácidos graxos de cadeia mais curta. Reações de escurecimento em alimentos incluem três reações não enzimáticas ─ Maillard, caramelização, e oxidação ─ e uma reação enzimática de escurecimento pela fenolase (Smith & Hui, 2004).

A deterioração dos alimentos também pode ser causada por fatores físicos, como temperatura, umidade e pressão. A umidade e o calor podem produzir hidrólise em gorduras, neste caso, as gorduras são divididas em ácidos graxos livres, que podem causar fortes odores e sabores rançosos em óleos e gorduras. O calor excessivo desnatura as proteínas e destroem os nutrientes, como vitaminas. No entanto, a baixa temperatura, como o congelamento, também descolore frutas e vegetais, a textura muda e seus revestimentos exteriores perdem a integridade e permitem a contaminação por microorganismos (Smith & Hui, 2004).

2.3.3 A conservação e o processamento de alimento

A deterioração dos alimentos pode ser prevenida por meio de práticas sanitárias adequadas na manipulação dos alimentos e processamento apropriado através de técnicas de preservação e condições de armazenamento padronizado. É evidente que os maiores responsáveis pela oferta maior de alimentos e segurança alimentar atualmente seja o processamento apropriado. Hoje, muitas técnicas são empregadas na conservação dos alimentos, tais como aditivos alimentares e tecnologias de processamento. Aditivos alimentares, entre outras funções, podem evitar a oxidação ao inibir ou destruir microorganismos nocivos (fungos e bactérias), e a vitamina C ou E pode servir como um antioxidante em muitos alimentos (Smith & Hui, 2004).

Varias tecnologias alternativas para conservação está disponível, entre elas o processamento térmico (calor seco, úmido, pasteurização) e não térmico (como microondas e pressão). No entanto, a radiação ionizante tem sido eficiente ao melhorar nossa habilidade de conservar os alimentos e, ao mesmo tempo, reduzir a incidência de algumas doenças. Embora a radiação tenha sido autorizada no processamento de várias categorias de alimentos, sua aplicação geral é ainda cuidadosamente regulamentada (Smith & Hui, 2004).

2.3.4 Efeito do processamento de alimento na estabilidade de alérgenos alimentares

A presença ubíqua de alérgenos na alimentação humana juntamente com uma maior sensibilização por meio da industrialização gerou a necessidade de medidas preventivas adequadas para proteger os consumidores sensíveis da exposição indesejada a esses alérgenos. As tentativas em reduzir ou eliminar alergenicidade de alimentos através do processamento de alimentos têm mostrado resultados variados (Sathe et al., 2005). Como a terapêutica atualmente disponível para tratar alergias alimentares não são tão efetivas, a melhor maneira de evitar a exposição involuntária a um alérgeno alimentar é a abstinência completa do alimento agressor. Por várias razões, evitar esse agressor nem sempre é possível, e, em certos casos, impossível. A rotulagem de alimentos precisa, em conjunto com boas práticas de produção, oferecer a segurança aos consumidores de uma maneira eficiente (Sathe et al., 2005).

No entanto, apesar das melhores intenções e práticas, a presença de agente vestigial não pode ser descartada em todos os momentos, a menos que métodos precisos estejam disponíveis para detectá-lo. Por estas razões, métodos específicos e sensíveis capazes de detectar quantidades mínimas de alérgenos alimentares ainda não foram desenvolvidos (Sathe et al., 2005). As relações entre a natureza dos epítopos alergênicos e os sintomas clínicos correspondentes, incluindo a gravidade, não são claros. Compreender essa relação é fundamental na concepção de formas de reduzir ou eliminar alergenicidade dos alérgenos (Sathe et al., 2005).

Pelo fato de, muitas vezes, os alimentos ou ingredientes alimentares serem sujeitos a uma variedade de condições de processamento, a alteração de epítopos podem potencialmente afetar as propriedades da proteína alergênica. O processamento pode destruir epítopo existente em uma proteína ou pode gerar novos, como resultado da

mudança na conformação da proteína. A formação de novos alérgenos foi reconhecida por pelo menos três décadas, e pode, parcialmente, explicar porque algumas pessoas podem tolerar alimentos frescos (*in natura*), mas não o correspondente processado (Sathe et al., 2005). Exemplo são os novos alérgenos descritos a partir de nozes (Malanin et al., 1995) e farinha de trigo processadas (Leduc et al., 2003).

Mais comumente, os métodos de tratamento têm sido associados com a diminuição da alergenicidade (fruto com pólen e alérgenos alimentares vegetais após aquecimento) ou sem efeito significativo (por exemplo, alérgenos termoestáveis de camarões após aquecimento). Epítopos conformacionais são tipicamente mais suscetíveis ao processamento por induzir a destruição do que os epítopos lineares. Epítopos lineares são alterados se forem hidrolisados. Alternativamente, epítopos lineares podem ser quimicamente modificados durante o processamento de alimentos ou ser intencionalmente alterados pela introdução de mutações através da Engenharia Genética (Sathe et al., 2005).

Como o processamento de alimentos envolve térmica, bem como tratamentos não-térmicos, cada tipo de tratamento pode variar no seu efeito sobre epítopos e, assim, tratamentos individuais devem ser considerados com cuidado ao avaliar a estabilidade antigênica. O processamento pode alterar alimentos de uma forma que permita atenuar epítopos, assim, reduzir ou aumentar o reconhecimento de alérgenos e, portanto, potencialmente alterar alergenicidade ao alimento agressor (Sathe et al., 2005).

A alteração na estrutura da proteína (pelo processamento) pode levar à modificação ou destruição dos epítopos, e, assim, comprometer positiva e negativamente a alergenicidade. Sanchez e Frémont (2003) têm amplamente analisado os efeitos do processamento de alimentos sobre a estabilidade estrutural de alérgenos alimentares e concluíram que os efeitos da interação proteína-proteína (especialmente agregação) são virtualmente desconhecidos.

Estudos sobre o efeito do processamento com calor úmido sobre peixes e fruta (kiwi) fornecem um exemplo interessante e particularmente ilustrativo do efeito sobre alergenicidade. Os achados sugerem que alguns dos principais alérgenos responsáveis por alergia alimentar IgE mediada para peixes e kiwi foram eliminados após tratamento térmico (Taylor et al., 2004; Fiocchi et al., 2004). Sen et al. (2002) demonstraram o papel da estrutura da proteína, particularmente a de pontes dissulfeto, na estabilidade proteolítica do

Ara h 2, o principal alérgeno em amendoins. Os investigadores relataram que a forma nativa bem como a desnaturada, quando *in vitro,* sofre hidrólise pela tripsina, quimiotripsina ou pepsina ainda mantêm a ligação com a IgE. Curiosamente, estes investigadores observaram mudanças substanciais na estrutura secundária de Ara h 2 com a redução de pontes dissulfeto. Ainda que essa mudança estrutural tenha ocorrido, não facilitou a proteólise de epítopos imunodominantes, sublinhando a importância de epítopos lineares em alergias.

2.4 Radioatividade

Foi descoberta casualmente por Antoine Henri Becquerel, em 1896, quando esse realizava experiências com um mineral do urânio. Becquerel observou que o mineral [K_2U0_2 $(S0_4)_2$. $2H_20$] era capaz de impressionar filmes fotográficos. Esses raios foram inicialmente denominados "raios do urânio". Em 1897, Marie Sklodowska Curie concluiu que era a quantidade de urânio que determinava a intensidade da radiação e, consequentemente, as mesmas radiações dependeriam do elemento (urânio). Em seguida, ficou provado que esse fenômeno se tratava de fenômeno atômico, ou, na realidade, fenômeno nuclear (Knool, 1979).

2.4.1 Radiação

O termo radiação vem do latim *radiare*, que indica um fenômeno básico em que a energia se propaga através do espaço, ainda que interceptada pela matéria. O termo irradiação vem do latim *in* e *radiare*, empregado para indicar o tratamento da matéria pela energia radiante. As radiações são produzidas por processos de ajustes que ocorrem no núcleo ou nas camadas eletrônicas, ou pela interação de outras radiações ou partículas com o núcleo ou com o átomo (Tauhata et al., 2003). Distinguem-se, assim, dois tipos de radiação: as chamadas corpusculares, feitas por intermédio de elétrons (raios beta), núcleos de hélio (raios alfa), núcleos de hidrogênio (prótons; p. ou H1) ou nêutrons (n ou n1); e as eletromagnéticas, constituídas pelos raios de comprimento de onda muito curto, os raios X e os raios gama (Pitorri, 1993). Enquanto os raios X são produzidos por geradores

especiais, os raios gama emanam espontaneamente de substâncias radioativas como rádio, tório e cobalto (Magalhães & Tauhata, 1994).

2.4.2 Medidas de intensidade das radiações ionizantes

A intensidade das radiações ionizantes é medida na base do número de íons que elas produzem num certo volume padrão. A unidade padrão é denominada Roentgem (R). No organismo humano, isso corresponde a cerca de duas ionizações por mícron cúbico (Knool, 1979). Em linhas gerais, as várias unidades de medida das radiações podem ser assim definidas:

- Roentgen (1 R), aplicado em tecidos moles, causa a absorção, por partes desses, de quantidade de energia igual a 93 ergs por grama. Um erg representa a energia desenvolvida pela massa de 1 grama, movendo-se com a velocidade de 1 centímetro por segundo;

- Rad, unidade empregada para raios alfa e beta, em tecidos moles, 1 R pode ser aceito como equivalente a 1 Rad;

- Gray (Gy), unidade empregada a qualquer radiação ionizante, quando submetida a qualquer material, representa a dose absorvida por um material no ponto P. 1 Gy equivale a 100 Rad.

2.4.3 Dose e taxa de dose

Dose é a quantidade total de radiação emitida; taxa de dose é a maneira como essa dose é distribuída ao longo do tempo. Assim, uma mesma dose (digamos 100 Gy), podendo ser aplicada durante diferentes períodos de tempo (1 minuto, 10 minutos, 100 minutos etc.), apresentar-se-á com diferentes taxas (de 100 Gy/min, de 10 Gy/min, de 1G y/min etc.), apesar de que, em todos os casos, a dose final de radiação emitida seja a mesma (100 Gy). Quanto maior a taxa e a dose, maior o dano (MS/S.N.V.S, 1992).

2.4.4 Efeitos biológicos gerais da radiação

É fácil compreender que a radiação ionizante pode agir sobre a célula e modificar a concentração de íons hidrogênio e o potencial de oxirredução de diferentes biomoléculas, alterando, profundamente, a funcionalidade molecular. As alterações químicas, decorrentes

da radiação ionizante, são realizadas por dois mecanismos: a) por ação direta ─ na qual a molécula sofre alterações, tornando-se ionizada ou excitada pela passagem de elétrons ou ondas eletromagnéticas; b) por ação indireta ─ na qual a molécula não absorve energia, mas recebe, por transferência, energia de outra molécula (Kempner, 2001).

O mecanismo pelo qual a radiação age sobre a célula é diverso, no entanto, os efeitos agudos possivelmente se devem à ionização da água. A água se decompõe e, como consequência, verifica-se a formação de compostos químicos ativos, que influenciam várias classes de biomoléculas (Tauhata et al., 2003). Os compostos que se formam são instáveis, de curta duração, mas seus efeitos podem ser profundos. Os principais representantes são os ânions superóxidos, radicais peróxidos, radicais hidroxil e peroxinitritos. Essa oxidação afeta facilmente grupos proteicos, em especial, o grupo sulfidril e sítios hidrofóbicos (Riley, 1994).

Em sistemas biológicos, os efeitos das radiações ionizantes diferem qualitativamente segundo a dose da radiação. Pequenas doses agem por ação indireta e produzem, principalmente, oxidações. Grandes doses agem por ação direta e indireta ao mesmo tempo. Vários fatores influenciam os efeitos radiobiológicos. Sendo especialmente importante: a) a intensidade da radiação; b) a temperatura de exposição c) a maneira da exposição, isto é, se simples, continuada ou fracionada; d) o tempo de exposição; e) presença de oxigênio (MS/S.N.V.S, 1992).

2.4.5 Aplicação biotecnológica da radiação gama

A aplicação de radiação gama para inativação de patógenos é evento crucial para iniciar a comercialização de produtos derivados de plasma humano em grande escala, sem o potencial risco de contaminação para receptores. Um método de inativação viral, usando irradiação gama em alta dose (45 kGy), provoca a inviabilidade de todo vírus presente no plasma e essa efetividade antiviral está diretamente relacionada ao dano no genoma viral (Miekka et al., 1998; Reid, 1998; Hiemstra et al., 1991). A irradiação foi, inicialmente, descartada como ferramenta para a inativação de patógenos no plasma e derivados por causa da pobre recuperação proteica. Pesquisas, envolvendo a proteção de proteínas contra os efeitos das radiações ionizantes, têm sido desenvolvidas com uso de antioxidantes (Zbikowska et al., 2006) e estratégicos modelos de exposição (Terryn et al., 2007).

2.4.6 Irradiação de alimentos

A irradiação de alimentos é uma tecnologia segura para reduzir as perdas de alimentos devido à deterioração, e para controlar a contaminação microbiológica e parasitária. A comercialização global de produtos irradiados ainda é pequena, apesar do conhecimento geral que pode oferecer todos estes benefícios, eliminando a necessidade do uso de substâncias químicas nocivas. Em parte, a indústria e a comunidade científica não foram bem-sucedidas na promoção da tecnologia e na educação do público. Digno de nota é o progresso que tem sido usado desde o início do século XX nas diferentes áreas, com regulamentação e harmonização internacional, bem como aplicações comerciais (GAO/RCED, 2000).

A era moderna na pesquisa de aplicações da irradiação de alimentos começou quando os Comissão de Energia Atômica dos Estados Unidos (USAEC) iniciou uma pesquisa para utilização de radiações ionizantes para preservação de alimentos em 1950 e começou a fornecer barras de combustível nuclear a partir de reatores nucleares. Já nas fases iniciais deste processo, a limitação nos gastos de barras de combustível tornou-se cada vez mais evidente, especialmente em relação à dosimetria exata.

Cobalto-60 (Co-60), um radioisótopo produzido deliberadamente, foi consideravelmente mais adequado para este fim. As fontes de cobalto-60 desde então tem sido amplamente utilizada desde 1960. No entanto, a pesquisa global em irradiação de alimentos continua. A irradiação de alimentos tem sido estudada mais do que qualquer outro processamento de alimentos. Todas as evidências recolhidas a partir de quase um século de investigação científica e técnica levam à conclusão de que a irradiação de alimentos é um processo toxicologicamente seguro, benéfico e prático (GAO/RCED, 2000).

Muitos anos de investigação resultaram em aprovações regulatórias para este processo em um número crescente de países. A comercialização de alimentos irradiados também está aumentando. Lojas de varejo que oferecem produtos irradiados para venda estão experimentando uma reação positiva do consumidor. Dada uma escolha livre e informações factuais, os consumidores estão escolhendo os alimentos irradiados (**Figura 13**). Importantes agências da ONU, como a Organização Mundial da Saúde e a Organização para a Alimentação e Agricultura, reconhecem a irradiação como um método

importante de controle de patógenos, da deterioração dos alimentos e importante método para controle fitossanitário de produtos agropecuários (GAO/RCED, 2000).

Figura 13. Símbolo internacional para identificação pelos consumidores de alimentos irradiados - a radura.

2.4.6.1 Irradiação de alimentos no Brasil

No Brasil, a legislação sobre irradiação de alimentos existe desde 1985 (Portaria DINAL no. nove do Ministério da Saúde, 08/03/1985). Entretanto, apesar desta regulamentação, custos elevados e pouco interesse governamental têm restringido aplicação desta tecnologia de conservação em nosso país. Até então, apenas duas empresas atuam comercialmente e estão localizadas no estado de São Paulo (CDTN, 2011).

O Centro de Energia Nuclear para Agricultura (CENA), da Universidade de São Paulo, vem realizando pesquisas na área e presta serviço para as indústrias. O Instituto de Pesquisas Nucleares, também da USP, além de realizar pesquisas na área, realiza um trabalho junto aos produtores, mostrando os benefícios e vantagens da irradiação de alimentos (CENA, 2011).

2.4.6.2 Benefícios e segurança da irradiação de alimentos

Os benefícios da irradiação de alimentos incluem: (1) redução de patógenos de origem alimentar; (2) aumento da vida útil de alimentos, retardando o processo de amadurecimento, e o surgimento de microorganismos; (3) controle de pragas de insetos e

parasitas, assim, reduzindo a necessidade de defensivos; (4) controle fitossanitário em alimentos exportados (CENA, 2011).

Vários organismos de controle e harmonização têm divulgado estudos que os compostos químicos produzidos nos alimentos irradiados são, geralmente, os mesmos em alimentos cozidos, e quaisquer diferenças não colocam consumidores em risco. Quanto à qualidade nutricional, os principais componentes dos alimentos, como carboidratos, proteínas e gorduras, sofrem mudanças mínimas durante irradiação, e a perda de vitaminas corresponde à mesma perda observada em alimentos cozidos, enlatados, ou mantidos em câmara fria (GAO/RCED, 2000, CENA, 2011).

2.4.6.3 Fontes de radiação usadas na irradiação de alimentos

Três tipos de radiação ionizante são regulamentados para irradiar alimentos: raios gama, elétrons de alta energia (por vezes referidos como feixes de elétrons) e raios-X. Até recentemente, os raios gama, especificamente os produzidos por cobalto-60, foram a fonte exclusiva da irradiação de alimentos nos Estados Unidos e na Europa. Enquanto os três tipos de radiação ionizante têm os mesmos efeitos sobre os alimentos, existem algumas diferenças na forma como eles trabalham. Por exemplo, feixes de elétrons e radiadores de raios-X são operados pela eletricidade e não usam isótopos radioativos (a exemplo do cobalto-60). No entanto, feixes de elétrons não podem penetrar em alimentos, como os raios gama ou raios-X, e, portanto, eles são usados, principalmente, para o tratamento de uma fina corrente de grãos (GAO/RCED, 2000).

2.4.6.4 Faixas de dose para aplicação em alimentos

Com base na dose de radiação, a aplicação é, geralmente, dividida em três categorias principais (FDA, 1997; CAC/RCP, 2003).

- Aplicações em baixa dose (até 1 kGy):
  - Inibição de esporos em bulbos e tubérculos (0,03-0,15 kGy);
  - Atraso no amadurecimento dos frutos (0,25-0,75 kGy);
  - Desinfestação de insetos, incluindo tratamento de quarentena e eliminação de parasitas de origem alimentar (0,07-1,00 kGy).

- Aplicações em média dose (1 kGy a 10 kGy):
  - Redução da deterioração microbiológica por prolongar a vida de prateleira de carnes, aves e frutos do mar sob refrigeração (1,5-3,0 kGy);
  - Redução de microorganismos patogênicos em carnes frescas e congeladas, aves e frutos do mar (3,0-7,0 kGy);
  - Redução do número de microorganismos em especiarias para melhorar a qualidade higiênica (10,0 kGy).

- Aplicações em alta dose (acima de 10 kGy):
  - Esterilização de carne embalada, aves e seus produtos que são estáveis em prateleira sem refrigeração (25,0-70,0 kGy);
  - Esterilização de dietas hospitalares e militares (25,0-70,0 kGy).

2.4.6.5 Irradiação de alimentos e a alergenicidade

Como defendido por Taylor no início da década de 1980, a prevenção da alergia alimentar pode ser alcançada por meio da alteração de fatores dietéticos responsáveis pela sensibilização e expressão fenotípica da doença. A partir de então, a hidrólise de alérgenos por enzimas proteolíticas e o desenvolvimento de alimentos recombinante com DNA modificado têm sido a esperança na eliminação dos alérgenos protéicos, quando comparado a tradicionais métodos de processamento (Nilsson et al., 1999). Entretanto, estas abordagens podem ser usadas apenas em limitados alimentos.

Enquanto isso, a modificação estrutural de proteínas dos alimentos por radiação foi observada (Kume et al., 1994) e esse resultado indicou que a radiação ionizante poderia mudar a antigenicidade pela destruição ou modificação epítopos conformacionais e lineares em alérgenos alimentares (Kume e Matsuda, 1995; Lee et al, 2000; Byun et al., 2002). Recentemente, a completa abolição da atividade intrínseca e perda da integridade estrutural com fragmentação e agregação após irradiação em ampla faixa de dose foram observadas em um estudo com lectina vegetal (Vaz et al., 2011). No entanto, nenhum estudo avaliou se a profunda modificação na estrutura molecular compromete a alergenicidade em modelos experimentais *in vitro e in vivo*.

2.5 O enovelamento e estabilidade proteica

O mecanismo pelo qual uma proteína se enovela a partir de conformações desordenadas é alvo de estudos há várias décadas. O entendimento desse processo envolve a caracterização de todas as espécies que ocorrem na reação de enovelamento e corresponde à oportunidade de relacionar a sequência de aminoácidos de uma proteína, sua estrutura, estabilidade e função biológica (Kumar & Yu, 2004). A cadeia polipeptídica de proteínas globulares é enovelada de forma compacta e esta conformação é importante para a função biológica e estabilidade estrutural. Isso foi evidenciado por experimentos de desnaturação proteica induzidos por aquecimento, exposição a valores extremos de pH ou adição de agentes desnaturantes, como ureia e Cloreto de guanidina (Kumar & Yu, 2004).

Quando uma proteína globular é desnaturada, seu esqueleto covalente permanece intacto, mas a cadeia polipeptídica desdobra-se ao acaso em conformações espaciais variáveis e irregulares. A maneira como as cadeias polipeptídicas são dobradas determina a estrutura terciária da proteína (Nelson & Cox, 2000). Esta pode ser estabilizada por quatro tipos de interação: pontes de hidrogênio entre as cadeias laterais, ou grupos R, de resíduos pertencentes a alças adjacentes, atração iônica entre grupos R com cargas elétricas opostas, interações hidrofóbicas e/ou interações covalentes.

Estudando o processo de enovelamento e desenovelamento proteico, vários pesquisadores verificaram que esse processo nem sempre ocorre em apenas duas etapas, como se pensava inicialmente. Foi identificada a presença de intermediários de enovelamento (Tanford, 1970).

O estado intermediário de enovelamento foi caracterizado por diversos pesquisadores que o descreveram como um estado compacto desnaturado, com significativo conteúdo de estrutura secundária, similar à estrutura nativa, e estrutura terciária flexível e desordenada (Barrick & Baldwin, 1993). Uma proteína em seu estado nativo, quando exposta às condições de estresse, como pH, temperatura, agente desnaturantes, entre outras, tende a se desenovelar e esse processo pode ocorrer em várias etapas, com a formação de um ou mais estados intermediários. O importante é que o equilíbrio esteja sempre deslocado no sentido da forma nativa, impedindo, assim, a formação de estruturas não funcionais e/ou que apresentem efeito tóxico (**Figura 14**).

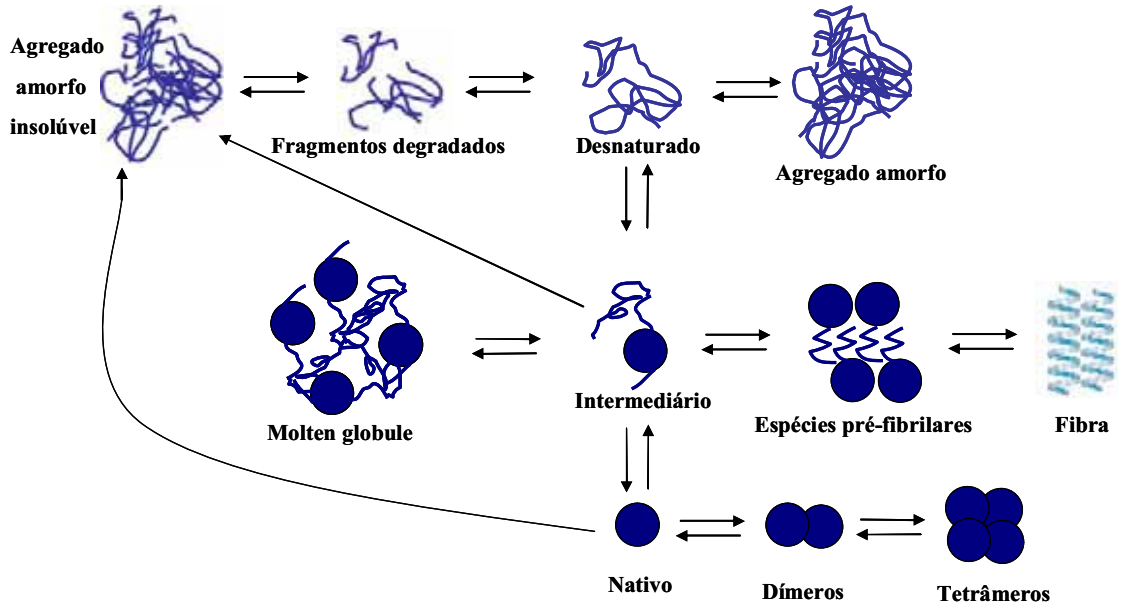


Figura 14. Representação esquemática de alguns dos estados conformacionais acessíveis à cadeia polipeptídica. As populações relativas dos diferentes estados irão depender da cinética e da termodinâmica dos diversos equilíbrios mostrados no diagrama. (Fonte: adaptado de Dobson, 2004).

Hoje, é sabido que uma proteína, ao se enovelar, pode passar por vários estágios cinéticos, com a formação de intermediários parcialmente enovelados no decorrer desta via (Baldwin, 1975; Wetzel, 1994). Com a melhor caracterização de espécies intermediárias do enovelamento, foi possível observar que, em muitos casos, os agregados são formados a partir de intermediários que se desviam da via normal de enovelamento e sofrem agregação (Christensen & Pain, 1991; Lai e cols., 1996; Kelly, 1998).

Pelo fato de alérgenos alimentares vegetais serem, em sua grande maioria, proteínas complexas, a estabilização de estruturas proteicas é uma questão importante a ser levantada, independentemente do âmbito em que o assunto esteja envolvido. Isso se deve ao fato de estruturas proteicas estarem susceptíveis a diversos tipos de alterações físicas e químicas, tais como proteólise, oxidação, agregação e mudanças conformacionais irreversíveis. O maior problema por trás de alterações conformacionais de proteínas é que estas nem sempre são perceptíveis a métodos gerais de análise, o que torna complexa a análise estrutural da dessas. Em virtude disso, normalmente, um grande conjunto de metodologias tem sido empregado: eletroforese, cromatografia, dicroísmo circular, fluorescência,

microcalorimetria e atividade intrínseca, dentre outras (UNITED States Pharmacopeia, 2005).

Esses conceitos foram empregados no desenvolvimento deste trabalho, contribuindo para a interpretação dos resultados apresentados.

3. Objetivos

3.1 Objetivos

Investigar e caracterizar como a irradiação gama compromete a estrutura molecular de alérgenos alimentares e sua antigenicidade ao acompanhar a resposta inflamatória alérgica de camundongos sensibilizados e submetidos a um agudo e crônico desafio oral.

3.2 Objetivos específicos

- Avaliar e caracterizar o efeito de radiação gama em ampla faixa de dose sobre a atividade intrínseca dos alérgenos selecionados para este estudo: Cramoll, Con-A e WGA;
- Correlacionar os efeitos observados sobre a estrutura molecular dos alérgenos e a irradiação, que envolve perda de atividade intrínseca, alteração na estrutura terciária e secundária, enovelamento, dentre outros parâmetros estruturais, em busca de maiores evidências do mecanismo comum pelo qual a radiação altera a relação estrutura-atividade em proteínas;
- Realizar ensaios de afinidade a fim de identificar a natureza dos agregados formados após irradiação empregando um marcador para superfície hidrofóbica;
- Explorar a constituição dos agregados formados após irradiação por meio de experimentos de perturbação da estabilidade proteica por variáveis químicas, como agentes caotrópicos (ureia), e por variáveis físicas, como temperatura com uso da Espectroscopia de Fluorescência, Dicroísmo Circular e Calorimetria Diferencial de Varredura;
- Averiguar a resposta inflamatória alérgica de camundongos sensibilizados e submetidos a um desafio oral agudo e crônico com alérgenos irradiados analisando a perda de peso, o perfil de leucócitos, os níveis plasmáticos das citocinas e alterações histológicas intestinais.

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5. Artigos submetidos à publicação

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High doses of gamma radiation suppress allergic effect of food allergen

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Abbreviations used
Cramoll: *Cratylia mollis* lectin
CD: Circular dichroism
Bis-ANS: 4.4'-bis-1-Anilinonaphthalene 8-sulphonate
HA: Hemagglutinating Activity
SHA: Specific HA
SDS–PAGE: Sodium dodecyl sulphate–polyacrylamide gel electrophoresis
DSC: Differential Scanning Calorimetry

Background: One of the most promising areas for the development of functional foods lies in the development of effective methods to reduce or eliminate food allergenicity, but few reports have summarized information concerning the progress made with food irradiation.

Objective: To investigate the relationship between allergenicity and molecular structure of a food allergen after gamma irradiation and to evaluate the profile of the allergic response to irradiated allergens.

Methods: Cramoll, a lectin isolated from a bean and used as a food allergen, was irradiated and the possible structural changes were accompanied by spectrofluorimetry, circular dichroism and microcalorimetry. Subsequently, sensitized animals subjected to intragastric administration of non-irradiated and irradiated Cramoll were treated for 7 days. Then, body weight, leukocytes, cytokine profiles and histological parameters were also determined.

Results: Cramoll showed complete inhibition of intrinsic activity after high radiation doses. Changes in fluorescence and CD spectra with a simultaneous collapse of the tertiary structure followed by a pronounced decrease of native secondary structure were observed after irradiation. After oral challenge, sensitized mice demonstrate an association between Cramoll intake, body weight loss, eosinophilia, lymphocytic infiltrate in the gut and Eotaxin secretion. Irradiation significantly reduces, according to the dose, the effects observed by non-irradiated food allergens.

Conclusion: We confirm that high-dose radiation may render protein food allergens innocuous by irreversibly compromising their molecular structure. However, our data also indicate that modulation of the gut immune response against the structural modification of food allergens should be further explored.

Key words: food allergy; food allergen; food irradiation; lectin.

Food allergy is defined as an immunologically mediated adverse reaction against dietary antigens. Food allergies can affect several organ systems, the symptoms commonly arising from the gut, skin and respiratory tract [1]. The prevalence of food allergies has increased dramatically in recent decades, due to the food consumed being increasingly processed and complex [2]. Food allergies are more common in pediatric patients than adults and have considerable medical, financial and social impacts on society. Food allergens are proteins belong to a variety of different protein families which resist proteolysis, harsh pH changes, or thermal treatments and can refold to their native structure upon cooling [3]

In food allergies, intestinal inflammation, disturbances in intestinal permeability and antigen transfer occur when an allergen comes into contact with the intestinal mucosa. Lectins are ubiquitous proteins of non-immune origin with the ability to recognize complex carbohydrates on cell surfaces [4] and are generally recognized as an important anti-nutrient of food [5]. Cramoll is a lectin isolated from *Cratylia mollis* seeds [6] from the same Leguminosae family and the same Diocleinae sub-tribe as Concanavalin A. Lectins can produce structural changes in the intestinal epithelium and resist gut proteolysis [7,8]. The emergence of several food and industrial proteins, as well as their potential to sensitize genetically predisposed populations to develop allergy, has prompted health officials and regulatory agencies around the world to seek approaches and methodologies to screen novel proteins for allergenicity [9,10]. Today, a large number of existing *in vivo* and *in vitro* tests are used for the diagnosis of allergies [11-13].

One of the most promising areas for the development of functional foods lies in the development of effective methods to reduce or eliminate food allergenicity. According to Sathe et al. [14], several methods to reduce immunological effects of food allergens have

been used. However some epitopes are likely to be more resistant to physical treatments and to be deactivated only by hydrolysis [15]. Despite this, biotechnology has contributed efficiently and safely to efforts to reduce food allergenicity [16]. Nevertheless, associated concerns about so-called cross-contact allergens which may arise from use of common or adjacent processing lines to prepare foods that may and may not contain any allergenic ingredients [17] cannot be eliminated by the incorporation of genetically modified foods.

Food irradiation is currently permitted by over 40 countries, and the volume of irradiated food is increasing annually world-wide [18]. The processing of food by ionizing radiation can change antigenicity of food antigens/allergens by two different means: first, by interacting directly on target proteins; and second, by the formation of major products from radiolysis of the water [19]. The exposure of proteins to radiation produces alterations to their physical and chemical structures, resulting in distortions of the secondary and tertiary structures [20].

Good manufacturing practices coupled with development of analytical methodology for the screening of allergenic ingredients are also relevant issues and cannot be replaced by incorporation of genetically modified foods. To our knowledge, very few reports summarize information concerning the progress made with gamma irradiation as a safe technology that can eliminate allergenicity in food. Thus, we investigated the relationship between allergenicity and protein modification after this type of physical processing. Furthermore, we believe that gamma irradiation has become a valuable tool, not only for the development of our knowledge on food allergy in general, but also as an alternative treatment to abolish allergenicity in increasingly processed and complex food.

METHODS

Materials

The broad-range molecular weight protein standard, Sephadex G-75, external fluorescence probe 4.4'-bis-1-anilinonaphthalene 8-sulphonate (bis-ANS) were purchased from Sigma Chemical Co., USA. Cramoll was purified as described by Correia and Coelho[6]. All solvents and other chemicals used were of analytical grade from Merck, Germany. All solutions were made with water purified by the Milli-Q system.

Samples irradiation

Aliquots of lectin in phosphate buffer (pH 7.2) in borosilicate glass vials (16-125 mm) were lyophilized and then irradiated dry under atmospheric O_2 by a Gammacell 220 Excel 60 Co gamma ray irradiator (Ontario, Canada) using doses of 1.0, 10.0 and 25.0 kGy at a rate of 8.25 kGy/h. Each dose was analyzed after irradiation by the following methods.

Hemagglutinating activity and protein concentration

Hemagglutinating activity (HA), which was defined as the lowest sample dilution that showed hemagglutination, was evaluated as described by Correia and Coelho [6]. Specific HA (SHA) corresponded to the relationship between the HA and protein concentration measured according to Lowry et al. [21] using bovine serum albumin (BSA) as the protein standard in the range of 0-500 µg/mL. The percentage of the remaining SHA ($\%SHA_{REM}$) was calculated according to the equation: $\%SHA_{REM} = (SHA)_{GM} / (SHA)_{GO} \times 100$, where G_M is the Cramoll SHA of each radiation dose (1; 10 and 25 kGy) and G_0 is the SHA of non-irradiated Cramoll (control).

Urea/SDS-PAGE

To detect any insoluble aggregates that formed, the precipitate was submitted to gel electrophoresis after centrifugation. SDS-PAGE was performed according to Laemmli [22]. To obtain complete dissolution of the aggregates, the samples were heated for 10 minutes at 60°C, then 5 M urea was added and heated for 10 more minutes at the same temperature.

Reverse phase chromatography analysis

To detect fragmented protein, the supernatant was separated by RP-HPLC after centrifugation. Irradiated samples were submitted to reverse phase chromatography on a C-4 column (Vydac-Protein Peptide

Ultrasphere) performed on an HPLC system (Shimadzu LC-10AD-Tokyo, Japan) and monitored at 280 nm. The column was equilibrated with 0.1% TFA (solvent A) and eluted using 90% acetonitrile/10% H_2O/0.1% TFA (solvent B) in a non-linear gradient, where B = 0% at t = 5 minutes; B = 45% at t = 10 minutes; B = 50% at t = 30 minutes and B = 100% at t = 35 minutes.

Fluorescence measurement

Intrinsic fluorescence assay was performed on a spectrofluorometer (JASCO FP-6300, Tokyo, Japan). The fluorescence emission intensity of tryptophan from irradiated Cramoll solution in phosphate buffer at pH 7.2 was measured at 25°C in a rectangular quartz cuvette with a 1 cm path length. For intrinsic fluorescence measurements, the excitation was at 295 nm and emission was recorded from 305 to 450 nm, using 5 nm band pass filters for both excitation and emission. The center of spectral mass (CM) were calculated according to the equation: $CM = \sum I_\lambda F_\lambda / \sum F_\lambda$, where F_λ stands for the fluorescence emission at wavelength I_λ and the summation was carried out over the range of appreciable values of F.

Hydrophobic surface analysis

The hydrophobic surface was measured using the same conditions as employed for the intrinsic fluorescence experiment. Samples were transferred to a quartz cuvette and then mixed with 5 µM bis-ANS. The fluorescence emission spectrum was obtained from 400 to 600 nm, with an excitation at 360 nm [23]. In order to see the fluorescence profile of denatured Cramoll, denaturation was performed with 8 M urea in phosphate buffer (pH 7.2).

Circular dichroism (CD) measurements

CD measurements were carried out on a spectropolarimeter (JASCO J-810, Tokyo, Japan). The protein concentrations were the following: non-irradiated (4 µM), 1 (4 µM), 10 (10 µM) and 25 kGy (20 µM) in phosphate buffer, pH 7.2 at 25°C. After irradiation, the samples were centrifuged and the measurements were performed with the supernatant. CD spectra were measured in the far-UV range (190-250 nm) in a 1 mm path-length quartz cuvette and in the near-UV range (250-320 nm) in 10 mm path-length quartz cuvette. The baselines (buffer alone) were subtracted from the protein spectra. Results were expressed as mean residue ellipticity, [θ], defined as $[\theta] = \theta_{obs}/(10.C.l.n.)$, where θ_{obs} is the CD in millidegrees, C is the protein concentration (M), l is the path-length of the cuvette (cm) and n is the number of amino acid residues assuming a mean number of 236 residues. The CDPro software package [24] was used to estimate the

fractions of secondary structures of Cramoll. The Cluster software was used to determine the tertiary structure class of Cramoll [25].

Thermal-stability and differential scanning calorimetry (DSC) analysis

Heat stability of the specific HA (SHA), for both irradiated and non-irradiated samples, was determined by incubation of the Cramoll solution (2.0 mg/mL) at different temperatures (30-100°C for 30 minutes) and the remaining SHA was determined in a pH of 7.2 at 25°C. The thermal stability of native and irradiated Cramoll was also determined by Differential Scanning Calorimetry (DSC). Thermal analysis was performed using a Microcal VP-DSC microcalorimeter (Northampton, MA, USA). Thermal transitions were analyzed by heating the sample from 5-100°C. A scan rate of 10ºC/h was used. The resulting thermograms were analyzed by the ORIGIN DSC software that was provided by Microcal Inc. (Northampton, USA).

Animals and treatment schedule

Female, albino Swiss mice (5 weeks old) were obtained from the breeding colony of the Departamento de Antibióticos da Universidade Federal de Pernambuco, Brazil. The animals were kept in an environmentally controlled room, temperature 21 ± 2°C, under a light/dark cycle of 12 hours, and were allowed free access to food and water. Requirements for care and handling of experimental animals according to international and Brazilian regulations (CEEA / UFPE n° 23076.033254/2010-14) were met. Albino Swiss mice were fed *ad libitum* with a Cramoll-free diet. All test substances were administered intragastrically by tube. Cramoll was dissolved in 0.5 mL saline (9 g NaCl/L).

Albino Swiss mice (*M. musculus*) was immunized subcutaneously on day 0; 15 and 30 using 0.5 ml Cramoll (10 µg/mL), dissolved in saline without use of an adjuvant (eight mice per group). Control animals were treated subcutaneously with 0.5 mL saline. Three days before starting the oral treatment in animals, they were stimulated with an intraperitoneal injection at the same dose. During one week, mice were treated for seven consecutive days: Non-immunized control animals were treated with 1 mL saline/day; Immunized mice were treated with 1 mL saline/day; Immunized mice were treated with non-irradiated Cramoll; and immunized mice were treated with irradiated Cramoll at 1, 10 and 25 kGy, respectively. The dose of bean lectin (27 mg/kg body weight)/day) was according to the total dietary intake of lectins in human subjects consuming vegetarian diets (calculations based on data from Peumans and van Damme [26].

Body weight and leukocyte evaluation

Body weight was determined before and after immunization and oral treatment. The final body weight of each group was obtained from the means of the individual values and expressed in grams (g). Blood samples were obtained and placed into micro-blood tubes containing the anticoagulant ethylenediamine tetra-acetic acid (EDTA). Hematological indices were determined by an automatic particle counter, the Coulter STK-S random-access clinical hematologic analyzer (Hospital das Clínicas, UFPE, Recife, Brazil).

Multiplex cytokine analysis

Multiple cytokine analysis kits were obtained from Genese Produtos Diagnósticos Ltda (São Paulo, SP, Brazil). Millipore multiscreen 96-well filter plates (Bedford, MA, USA) were used for all multiplex cytokine kits. Assays were run in triplicate according to the manufacturers' protocol. Data were collected using the Milliplex Analyser 200 version 2.3 (Luminex, Austin, USA). Data analysis was performed using the software Analyst version 3.1.

Histology

After 7 days of oral administration, the mice were sacrificed by decapitation. The histopathological evaluation of organs (jejunum, liver and spleen) of animals was performed with an optical microscope. Histological sections of 5µm were fixed and stained by routine histologic hematoxylin and eosin (HE) and mounted between slide and coverslip with synthetic resin. After mounting, the preparations were evaluated using a video-microscopy system (MOTIC BA200 Microscope, digital camera with Motic 1000-1.3M Pixel USB 2.0).

Statistical analysis

Differences between treatment groups of *in vivo* experiments were performed using one-way analysis of variance (ANOVA), followed by the *post hoc* Bonferroni test, with a significance level of $p < 0.05$, using the GraphPrism® program (GraphPad Software Inc., San Diego, CA, USA).

Results

Activity and molecular analysis

The three dose categories for processing of food by ionizing radiation were selected in the study. The comparison of the specific hemagglutinating activity (SHA) of Cramoll in its native (non-irradiated) and irradiated forms revealed major changes at the high dose of radiation (Figure 1A). Similar behavior was also observed at 1 kGy in which SHA was reduced by approximately 50%. To evaluate the circumstances of the loss of HA, gamma-treated samples were centrifuged, and the supernatants and precipitates were then subjected to SDS-PAGE. Non-irradiated samples showed three major components, with relative mass of 30 (intact protein), 16 and 14 kDa. A typical SDS-PAGE profile of the gamma-treated samples is shown in Figure 1B. The initial degradation of lectin was observed at 1 kGy. The appearance of high molecular weight components (Figure 1B - arrows) suggests that ionizing radiation may induce peptide bond cleavage as well as fragments of proteins which suffer later aggregation.

To detect the nature of insoluble aggregates, the precipitate was studied using urea/SDS PAGE after centrifugation and the supernatant was analyzed by RP-HPLC. The samples in the presence of the chaotropic agent (5 M urea) showed dissociation of the aggregates, suggesting that the irradiation induced the rupture of covalent bonds with complete fragmentation of the polypeptide chains at 25 kGy (Figure 1C). Non-irradiated Cramoll was separated by RP-HPLC into three components, p1, p2 and p3 (intact protein). The reverse-phase chromatography analysis revealed a loss of the peak area after irradiation (Figure 1D). No changes were detected after exposure to a low dose of radiation (1 kGy). Degradation of the main (p3) and secondary peaks (p1 and p2) were detected at doses above 10 kGy, as indicated in Figure 1D. In order to clarify that the aggregates are

composed of low molecular weight fragments, we decided to further investigate the nature of the aggregates formed after irradiation.

Structural analysis

The change in tertiary structure of Cramoll as a function of irradiation can be followed by measuring the fluorescence spectra. The intrinsic fluorescence emission did not change (at 1 kGy) and decreased (at 10-25 kGy) while changing the λmax, at approximately 8 nm for tryptophan residues. As shown in Figure 2A, spectral changes occurring in the 335 nm region result primarily from changes in the environment of the aromatic indole chromophores and phenol and are proportional to the degree of irradiation. Bis-ANS fluorescence was weakly observed in the non-irradiated Cramoll at 515 nm. After irradiation, bis-ANS fluorescence increased for all doses, while the peak shifted to 498 nm and its intensity increased (Figure 2B). We also noticed in all measurements that bis-ANS fluorescence in the absence of Cramoll or in the presence of its denatured form after treatment with 8 M urea is insignificant (data not shown).

Figure 3A shows the far-UV spectrum of non-irradiated Cramoll. This spectrum presents a stronger positive maximum at 198 nm and a single minimum negative at 222 nm. Additionally, the Cluster program classified Cramoll as an all-β class protein. The fractions of secondary structure estimated for native Cramoll were 50% β-sheet, 21% β turn, 5% α-helix and 24% unordered form with a root mean square (RMS) lower than 3%. Far-UV CD spectra of the irradiated samples at various doses (Figure 3A) decreased the CD intensity at 198 nm and the negative ellipticity values at 222 nm, resulting in a decrease of β structure with a concomitant increase of the random coil (≈ 40% at 25 kGy). Non-irradiated Cramoll showed a near-UV CD spectrum characterized by several peaks and shoulders which reflect

the tertiary structure in which the aromatic amino acids are inserted and indicate that the protein is folded into a well-defined structure (data not shown). The dose of 1 kGy causes the accommodation of the tertiary structure to decrease for Trp (around 290 nm) and Phe bands (≈ 260 nm) in near-UV. For irradiated samples at 10 and 25 kGy, the measurements in the near-UV were not run due to higher protein aggregation.

In order to evaluate the effect of gamma irradiation on thermal stability, we used HA and microcalorimetry as a function of temperature variation. Non-irradiated Cramoll was stable up 70°C and an increase of temperature above 70°C resulted in loss of activity. However, irradiated samples lost SHA below 60°C (data not shown). Thermograms of Cramoll are shown in Figure 3B. The calorimetric scan of non-irradiated samples was characterized by a pre main transition region from around 11 to 60°C and a main transition showing a single peak centered at 85°C. Gamma-treated samples were also submitted to DSC analysis and it was possible to observe the absence of a main transition peak. However, the pre-transition region became more intense than those for the non-irradiated protein. All the thermal denaturation transitions were found calorimetrically irreversible as reflected by the lack of transition in the second run of all the samples. For samples irradiated at 10 and 25 kGy, this analysis was not run due to higher protein aggregation.

Treatment schedule

After oral administration with saline, sensitized but not non-sensitized mice lost body weight. Moreover, mice showed weight loss in non-irradiated and irradiated Cramoll groups, when sensitized and exposed to the antigen solution by an oral route. A complete summary of results from all experiments is shown in Table I. The results also indicated that the non-irradiated Cramoll group showed less body weight when compared to groups

irradiated at 1, 10 and 25 kGy. The average leukocyte count of the non-sensitized, sensitized, non-irradiated and irradiated groups were not different. However, sensitized animals subjected to intragastric administration of non-irradiated Cramoll showed a significant difference in the average number of lymphocytes ($p < 0.05$), neutrophils ($p < 0.05$) and eosinophils ($p < 0.05$) when compared to non-sensitized and sensitized groups.

Non-irradiated Cramoll significantly increased production of Eotaxin and Rantes ($p < 0.05$) in sensitized albino Swiss animals after 7 days of continuous administration. However, mice showed no significant increase in IL-4 and IL-5, even when sensitized and exposed to the antigen solution by an oral route (Table I). Non-irradiated Cramoll ingestion induced an increase in lymphocytic infiltrate in the stroma of the microvilli and also around the tubular glands or crypts with numerous eosinophils in the proximal jejunum of sensitized mice compared to non-sensitized animals (Figure 4 A-D). Histopathological evaluation of liver and spleen showed no change even after oral challenge with non-irradiated Cramoll (data not shown).

Likewise, Cramoll ingestion also induced increase in Eotaxin and IL-5, when subjected to irradiation at a low dose (1 kGy). The comparison of non-irradiated and irradiated groups revealed similarities in the histological analyses (see Figure 5 A-F). However, the comparison of irradiated Cramoll at 10 kGy revealed significantly lower amounts of eotaxin secretion, when compared to non-irradiated Cramoll (Table I). In addition, the ratio of lymphocyte infiltration in the stroma of the microvilli and also number of eosinophils was much smaller (Figure 5 C-D). With respect to the secretion of IL-4, IL-5, and Rantes no significant differences can be found between these groups (Table I). Moreover, irradiated Cramoll at 25 kGy showed significant reductions of Eotaxin and lymphocyte infiltration in microvilli, compared to previous treatments (Figure 5 E-F).

Discussion

Although basic research on the structural changes in proteins caused by gamma radiation has been going on for a number of years, clinical research on the effect of such changes on food allergens remain poorly characterized. In particular, the effects of these irradiated allergens have not been examined *in vivo*. Therefore, it is unknown whether irradiated food allergens differ from non-irradiated allergens in their ability to produce allergic responses. In this study, we analyzed the effect of radiation on the structure and activity for a major Camaratu bean allergen, Cramoll. We further used this lectin to directly examine the interference in allergic response of mice which were sensitized and subjected to intragastric administration of non-irradiated and irradiated Cramoll.

The scarcity of detectable SHA in the majority of irradiated Cramoll suggests that there have been changes in the environment of the carbohydrate recognition domains. As shown by Vaz et al. [27], activity changes occurring as a result of high doses of radiation result primarily from changes in the environment of the carbohydrate recognition domains. Three dose categories used for the processing of food by ionizing radiation have been shown to change the structural basis for selective sugar recognition and binding, which has revealed that primary binding sites of leguminous lectins were impacted.

As shown by De Souza et al. [28], N-terminal sequence determination of the lighter components of Cramoll indicated that they corresponded to the N-terminal portion p1 (16 kDa) and the carboxyl terminal portion p2 (14 kDa) of the intact protein p3 (30 kDa). Because p1 and p2 are extremely insoluble [28], possibly they were removed from the supernatant after centrifuging and could not, therefore, be detected by HPLC. However, when we observed the urea/SDS-PAGE profile it was not possible to detect lighter components, thus indicating complete fragmentation of the polypeptide chains. Irradiation

caused a breakdown of polypeptide chains and also resulted in the formation of high molecular weight molecules. This aggregation occurs through inter-protein cross-linking reactions, hydrophobic and electrostatic interactions [29].

The emission spectrum of irradiated Cramoll is consistent with data of red-shifted emission spectra suggesting thermal and chemical denaturing as found for other legume lectins [30]. This finding was different from that reported by Vaz et al. [27] in which there was no observed shift in spectral center of mass after irradiation. Because we used a dry irradiation procedure, the effect, via water radiolysis, which produces reactive oxygen species that are responsible for hydrogen abstraction from amino acid side chains, rings of aromatic residues [31] were not observed in our study.

Binding of the hydrophobic fluorescent probe, bis-ANS, to proteins occurs upon the exposure of hydrophobic clusters during the unfolding process. Obviously, there are large clusters of solvent exposed to hydrophobic regions that are unveiled during denaturation and that are impinged by molecules of bis-ANS, with a resultant increase in its emission intensity and a blue shift of its emission maximum. In other words, the conformations that exhibit strong binding of bis-ANS seem to contain native secondary structures and no tertiary structure and, thus, show properties of a molten globule state [32]. Such intermediate species with similar spectral losses have also been observed in chaotropic denaturing of peanut agglutinin [33]. On the other hand, the fluorescence and far-UV CD can also provide important evidence for the nonexistence of molten globule states in Cramoll irradiated, since in high doses (10-25 kGy), it retains neither tertiary nor secondary structure, which indicates the existence of precipitation into insoluble amorphous aggregates with a lack of native β-rich secondary structures.

Previously described methods to reduce unwanted or intolerant immunological effects of food allergens have been inefficient [14] because many such allergens are resistant to cooking and digestive enzymes. DSC is a powerful technique that makes it possible allows to obtain valuable information on thermodynamics and kinetic features of the thermal unfolding of proteins [34]. The transition proceeds in a two-state manner, i.e., the concentration and constitution of intermediates between the native (non-irradiated) and the denatured state (irradiated) are evidently distinguished. However, the Cramoll at a dose of 1 kGy can be also easily fragmented by heat treatment.

Gamma rays are penetrative and their actions on food allergens can making them harmless, even after food processing and packaging. Currently, little is known about how processing may alter food allergens, and hence there is a need to systematically investigate the relationship between food protein allergenicity and the effect of food irradiation. Processing was shown to destroy epitopes of a protein and generate new ones (formation of neoallergens) as a result of conformational changes [14]. A decrease or increase of allergenicity can be caused by protein unfolding, misfolding or aggregation as well as by chemical modifications after food irradiation.

The sensitization procedure alone did not induce weight loss and changes in leukocyte count. However, experiments clearly demonstrate an association between non-irradiated Cramoll intake, body weight loss and eosinophilia. Therefore, the increased weight loss by food allergic disorders typically affect the gastrointestinal tract with different degrees of eosinophilic inflammation, edema and weight loss has been observed [35]. Such correlations were not found in irradiated Cramoll at radiation doses of 10 and 25 kGy.

However, irradiated Cramoll at low doses do not attenuate the allergic response, by contrast, they can make it even more offensive. We also conclude that the structural change experienced by Cramoll at 1 kGy was not sufficient to eliminate the antigenic character of such food allergens. As shown by Sathe et al. [14] low doses of radiation can destroy epitopes and generate new ones (formation of neoallergens) as a result of conformational changes on the protein. However, the destruction of epitopes as a result of conformational changes of the protein cannot be accelerated by low dose radiation.

The augmentation and/or maintenance of gastrointestinal inflammation in food allergies are controlled by the secretion of cytokines from antigen-stimulated CD4+ T cells. Allergic inflammation is driven by the T helper 2 (Th2) class of T cells. These cells generate the cytokines IL-4, IL-5 and IL-13. These cytokines play important roles in regulating eosinophil recruitment into the gut [36]. The low number of eosinophils observed in the gut, even when sensitized and exposed to the antigen solution by an oral route, provides evidence that IL-4 and IL-5 did not produce a large infiltration. Furthermore, according to De Melo et al. [37], Cramoll induces high levels of IFN-γ, and thus, it shows a typical response of Th1-type T cells. The low secretion of IL-4 and IL-5 with low eosinophil recruitment, after intragastric administration of non-irradiated Cramoll may be due to their specific response through Th1-type CD4 + T cells.

Chemokines have been implicated in both inflammatory and homeostatic leukocyte migration [38-40]. Eotaxin and Rantes secretion was stimulated after animals were sensitized and exposed to the antigen solution. Eotaxin, by contributing to the recruitment of Th2 cells to the allergic site, promotes the initiation and maintenance of an allergic reaction. As expected, given the findings of amorphous aggregate in irradiated Cramoll at 25 kGy, we observed a reduction in the secretion of the major chemokine (eotaxin), lower

degrees of lymphocytic infiltrate and eosinophilic number in the proximal jejunum of sensitized animals subjected to intragastric administration.

Importantly, our study describes the relationship between allergenicity and food allergen modification after food irradiation. Indeed, it provides important evidence for the absence of structural integrity in irradiated food proteins, since at high doses, the allergens do no retain tertiary or secondary structure, which indicates the existence of precipitation into insoluble amorphous aggregates with a lack of native conformational structures. Thus, we believe that high-dose radiation may render innocuous protein food allergens in a direct and irreversible form. In order to further prove the importance of food irradiation, the development of experimental allergy *in vivo*, via sensitized mice subjected to intragastric administration of irradiated food protein showed a reduction of intestinal inflammation observed in allergic responses to dietary antigens. Food protein allergens are associated with intestinal stress, suggesting than an allergen stimulus may also induce eotaxin secretion. It is important to note that the level of lymphocytic infiltrate in the stroma of the microvilli correlated with the eosinophil counts in the epithelial basal layer was reduced after irradiation of the food allergen. While data collected from animal models cannot be directly applied to humans, this experiment emphasizes the crucial role of radiation in tackling food allergens. Our results highlight the need to further characterize the involvement of irradiated food allergens on modulation of the gut immune response toward inflammation, profile of cytokine, immunoglobulin production and focus attention on the potential benefit of food irradiation as an alternative treatment to abolish allergenicity in food.

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Conflict of Interest

The authors declare that there are no conflicts of interest.

Capsule summary

- Currently little is known of how processing may alter allergens. This article reveals structural changes of a food allergen after food irradiation which can reduce allergenicity and help control the increasing incidence of food allergies.

Key messages

- High-dose radiation profoundly alters the structure of food protein allergens.
- Food irradiation may render food allergens innocuous in a direct and irreversible form.

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Figure captions

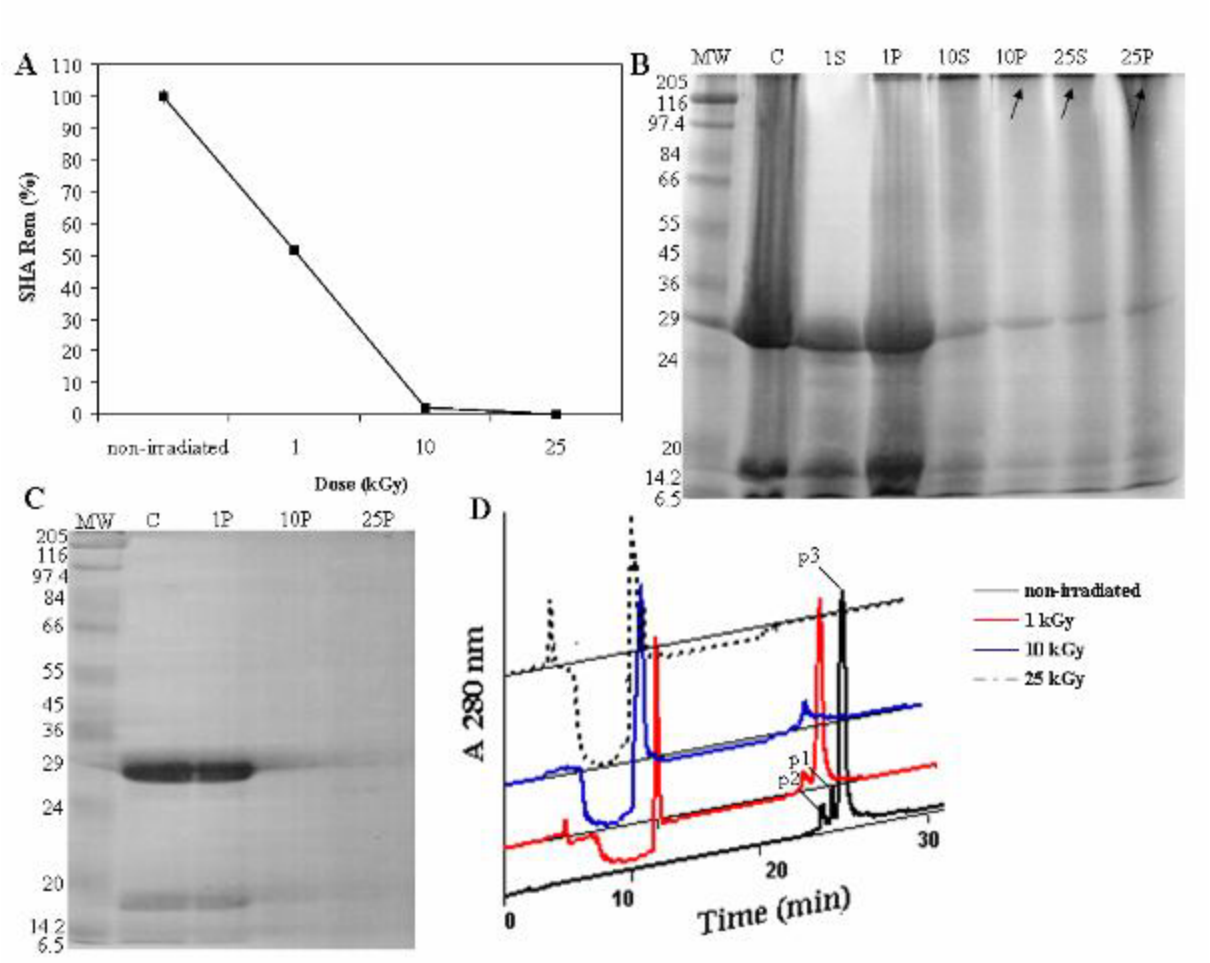
Figure 1 - Effect of γ-radiation on the activity and molecular structure of Cramoll. (**A**) $\%SHA_{REM}$. (**B**) SDS–PAGE. (MW) Molecular weight; (C) non-irradiated; (1P) 1kGy precipitate; (1S) 1kGy soluble fraction; (10p) 10kGy precipitate; (10s) 10kGy soluble fraction; (25p) 25kGy precipitate; (25s) 25kGy soluble fraction. (**C**) SDS-urea gel. (**D**) Reverse phase chromatography in an HPLC system.

Figure 2 - Cramoll intrinsic and Bis-ANS fluorescence. (**A**) Center of spectral mass of intrinsic fluorescence. (**C**) Center of spectral mass of Bis-ANS fluorescence.

Figure 3 - Far-UV CD spectra and DSC thermograms of Cramoll. (**A**) CD spectra were measured in the Far-UV range (190-250 nm). (**B**) Cramoll thermal denaturation monitored by DSC.

Figure 4 - Jejunum of untreated and treated mice (**A**) Non-immunized mice: Microvilli (**mv**) coated high enterocytes (**e**); HE 400x. (**B**) Leukocyte infiltration; HE 1000x. (**C**) Mice treated with non-irradiated Cramoll: there is a lymphocytic infiltrate in the stroma of the microvilli (**mv**) and in the colon zone (**cz**); HE 400x. (**D**) Leukocyte infiltration in submucosa (**arrow**) with numerous eosinophils (**eo**) HE 1000x.

Figure 5 - Jejunum of mice treated with irradiated Cramoll. (**A**) Mice treated with irradiated Cramoll (1 kGy) - HE 400x: microvilli (**mv**); tubular glands or crypts (**tb**) with numerous (**eo**) eosinophils; HE 1000x (**B**). (**C/D**) Mice treated with irradiated Cramoll (10 kGy); HE 400x: (**eo**) eosinophils; HE 1000x. (**E/F**) Mice treated with irradiated Cramoll (25 kGy): microvilli (**mv**) and tubular glands (**tb**).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SHA Rem (%)
non-irradiated
1
10
25
Dose (kGy)
B
MW
C
1S
1P
10S
10P
25S
25P
205
116
97.4
84
66
55
45
36
29
24
20
14.2
6.5
C
MW
C
1P
10P
25P
D
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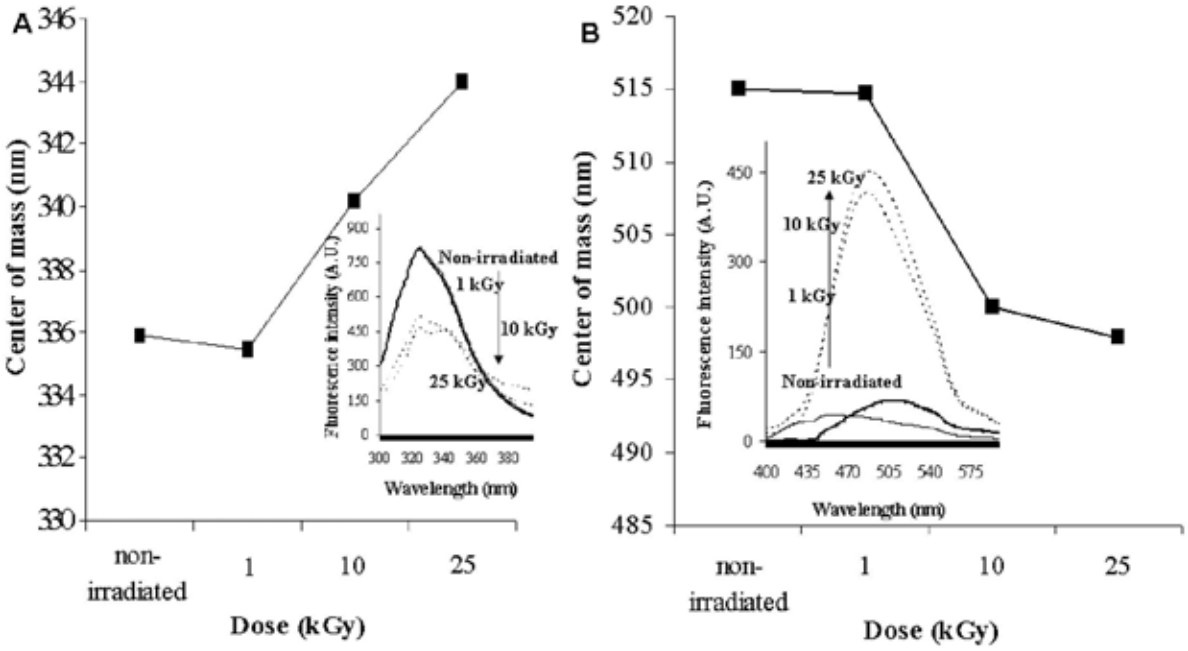
p2
p3
Time (min)
non-irradiated
1 kGy
10 kGy
25 kGy

Figure 1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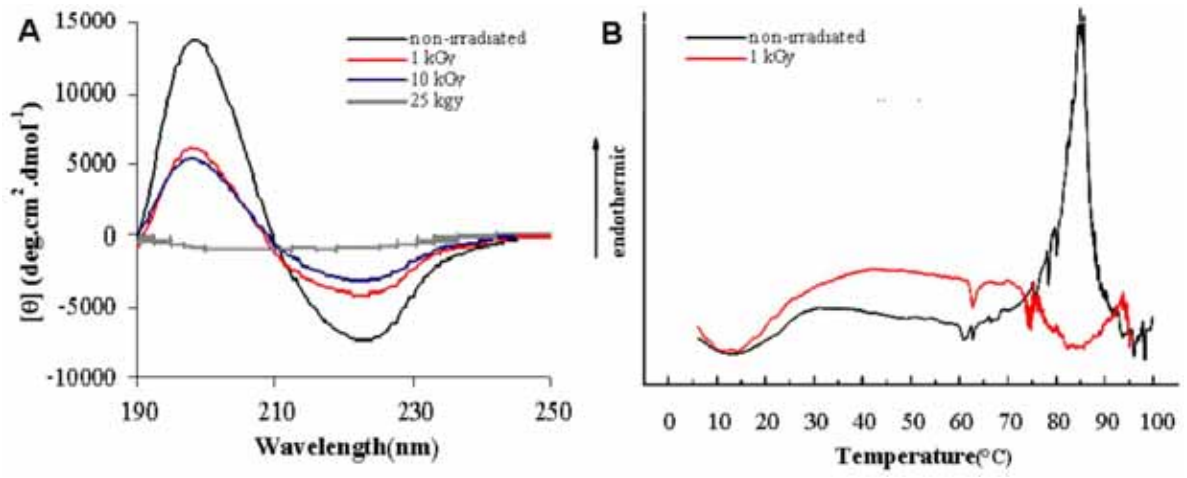
Center of mass (nm)
Dose (kGy)
non-irradiated
Fluorescence Intensity (A.U.)
Wavelength (nm)
Non-irradiated
1 kGy
10 kGy
25 kGy
B
Center of mass (nm)
Dose (kGy)
non-irradiated
Fluorescence Intensity (A.U.)
Wavelength (nm)
25 kGy
10 kGy
1 kGy
Non-irradiated

Figure 2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10000
5000
0
-5000
-10000
[θ] (deg.cm².dmol⁻¹)
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210
230
250
Wavelength(nm)
non-irradiated
1 kGy
10 kGy
25 kgy
B
non-irradiated
1 kGy
endothermic
0
10
20
30
40
50
60
70
80
90
100
Temperature(°C)

Figure 3

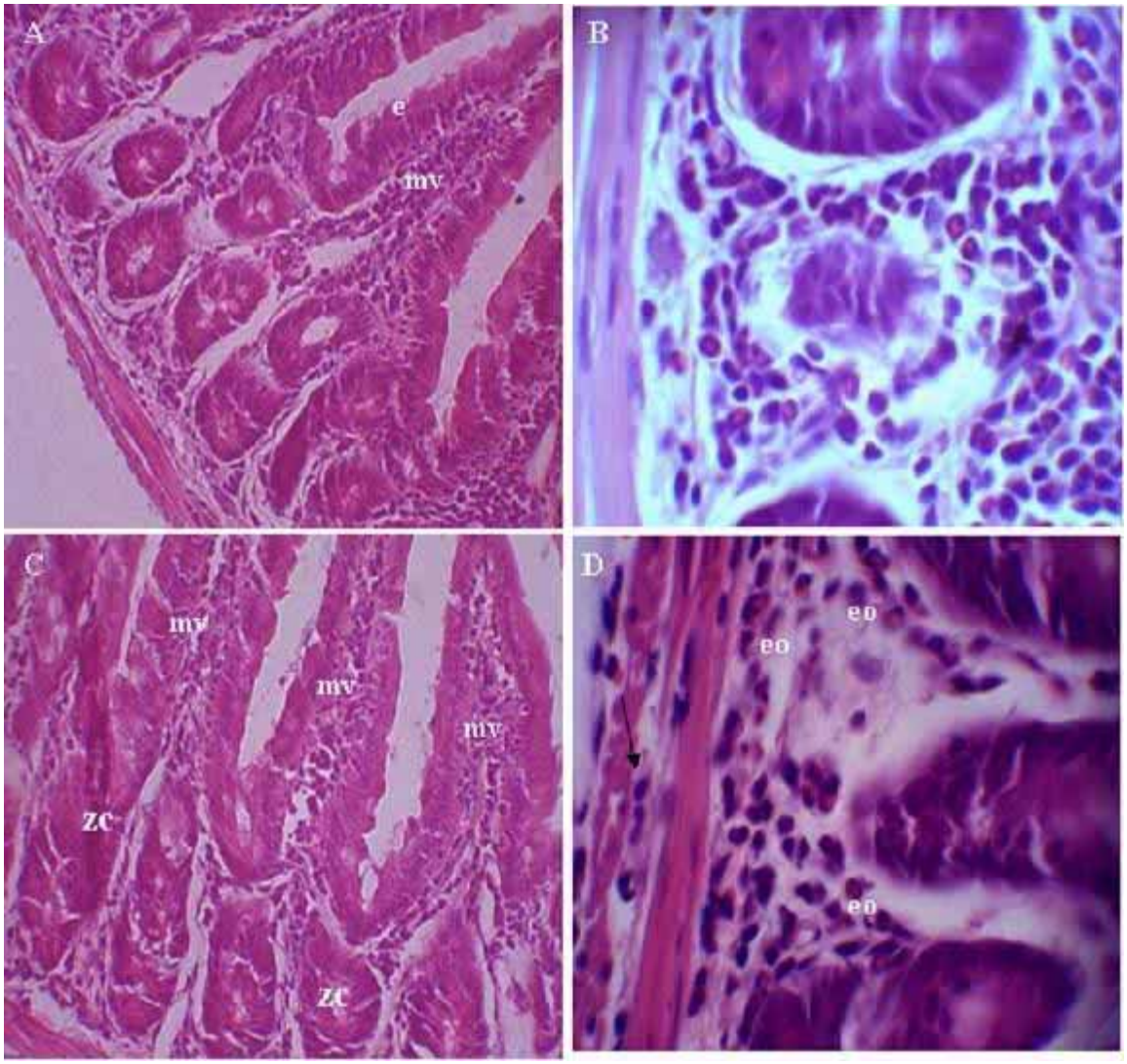
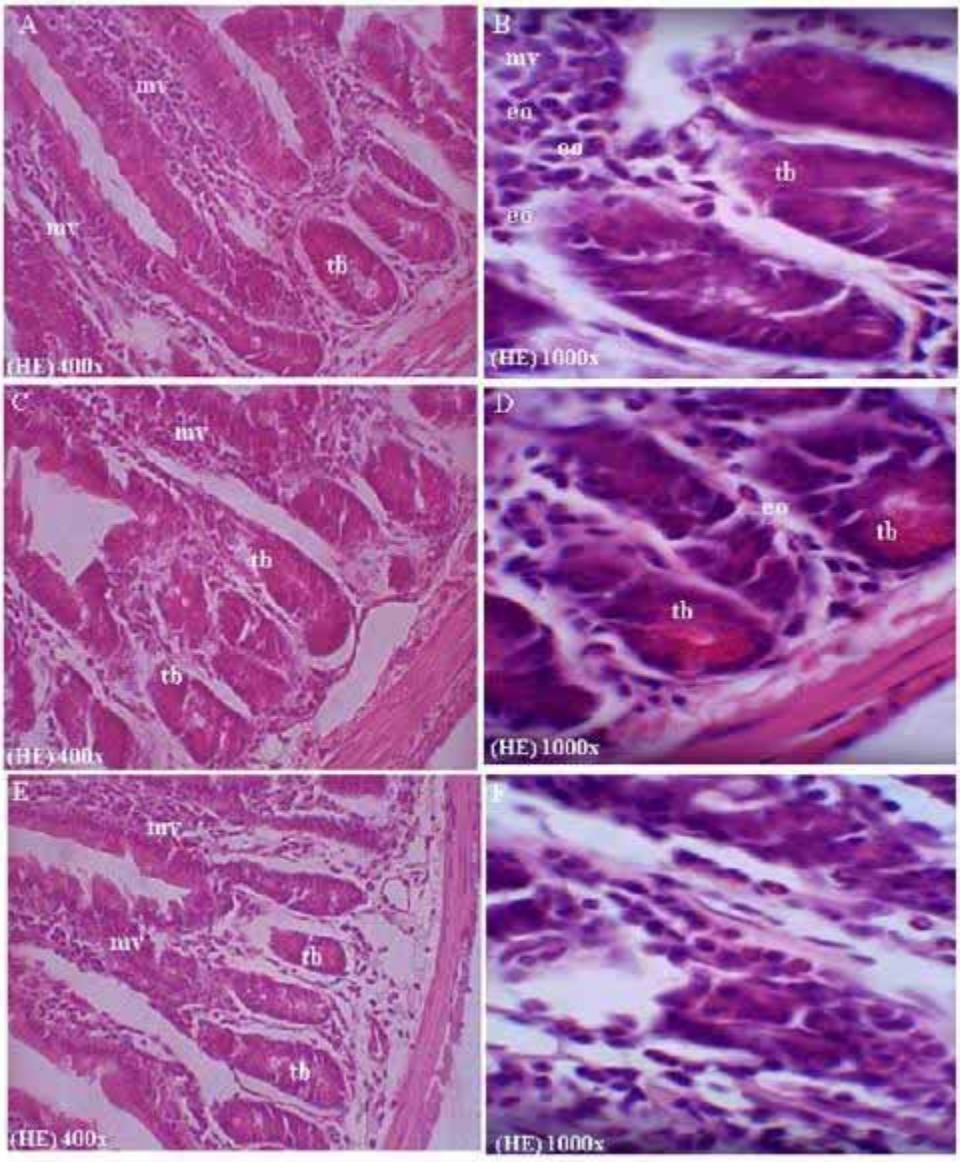


Figure 4


A
mv
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(HE) 400x
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C
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D
eo
th
th
(HE) 1000x
E
mv
mv
th
th
(HE) 400x
F
(HE) 1000x

Figure 5

Table I - Body weight, leukocytes and cytokine profiles of sensitized mice.

		Groups					
Weight (g)	**Number of mice**	**Non-immunized control**	**Immunized mice treated (saline)**	**Non-irradiated Cramoll**	**Irradiated Cramoll (1 kGy)**	**Irradiated Cramoll (10 kGy)**	**Irradiated Cramoll (25 kGy)**
Initial	8	27.8 ± 2.2	28.3 ± 1.3	24.9 ± 0.3	22.2 ± 2.1*	26.1 ± 0.4	26.0 ± 0.3
Post immunization	8	42.1 ± 1.7	37.1 ± 2.5*	31.5 ± 1.7*#	32.0 ± 4.7*#	35.8 ± 2.1*	35.9 ± 3.4*
Post treatment	8	43.9 ± 1.8	36.9 ± 1.8*	29.8 ± 1.4*#	31.0 ± 4.7*#	35.0 ± 1.6*	34.0 ± 3.6*
White blood count							
WBC[a]	8	3.2 ± 0.7	1.9 ± 0.7	4.8 ± 1.7	4.3 ± 1.5	5.5 ± 0.8	4.8 ± 1.1
Large lymphocytes[b]	8	84.0 ± 14.6	78.0 ± 13.5	53.5 ± 15.9*#	77.3 ± 7.3$	82.4 ± 4.1$	78.8 ± 8.6$
monocytes[b]	8	1.8 ± 1.3	3.4 ± 3.0	1.7 ± 0.1	3.9 ± 0.9	2.4 ± 1.9	2.4 ± 1.5
Neurophils[b]	8	11.4 ± 2.3	16 ± 2.5	35.1 ± 14.3*#	16.3 ± 6.4$	14.8 ± 3.1$	18.4 ± 8.1$
Eosinophila[b]	8	0.7 ± 0.1	2.6 ± 0.7	9.7 ± 3.8**	2.3 ± 1.1	0.4 ± 0.2	0.4 ± 0.1
Cytokines and chemokines							
Eotaxin (pg/mL)	8	523.7 ± 115.2	1003.0 ± 79.7	1960.0 ± 499.5*#	2066.1 ± 687.7*#	1183.4 ± 278.9	1144 ± 250.7$&
IL-4 (pg/mL)	8	< 0.3	30.1 ± 21.6	26.6 ± 11.9	32.1 ± 11.6*	33.4 ± 26.9	23.5 ± 12.7
IL-5 (pg/mL)	8	14.4 ± 6.8	29.8 ± 9.1	108.8 ± 86.0	135 ± 70.83*	54.1 ± 10.6	151.8 ± 135.6
Rantes (pg/mL)	8	34.5 ± 7.4	56.9 ± 22.6	110.4 ± 40.1*	76.32 ± 39.1	64.2 ± 60.8	63.8 ± 22.7

[a]White blood count in thousand per cubic millimeter. [b]Mean per cent of total leukocytes. *$p < 0.05$ compared to non-immunized control animals; #$p < 0.05$ compared to immunized mice treated with saline; $$p < 0.05$ compared to immunized mice treated with non-irradiated Cramoll; &$p < 0.05$ compared to immunized mice treated with irradiated Cramoll at 1 kGy.

Capítulo 2

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Gamma irradiation of food allergen at a low dose increases its allergenicity in a chronic food allergy model

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Abstract

Background: Few chronic food allergy models have described the relationship between allergenicity and the molecular structure of food allergens after food irradiation. Here, we established a chronic allergic mice model to clarify the profile of the allergic response to allergens irradiated at low and high doses.

Methods: The effect of γ-radiation on the structure of the allergen was measured by fluorescence, circular dichroism and microcalorimetry. BALB/c mice were intraperitoneally sensitized and then given non-irradiated and irradiated Con-A by daily gavage for 28 days. Body weight, leukocytes, cytokine secretions of IL-4, IL-5, Eotaxin and RANTES were examined and the morphological changes and lymphocyte infiltration in the small intestine was observed by using an optical microscope.

Results: The tendency to form insoluble amorphous aggregates and partially unfolded species was observed after irradiation. The administration of non-irradiated and irradiated samples at low-dose significantly increased weight loss as well as plasma levels of eotaxin in animals repeatedly exposed to Con-A. Significant lymphocytic infiltrate filling completely the stroma of microvilli and tubular glands was observed in the small intestinal of the group given Con-A irradiated at a low dose. This phenotype was not observed in animals treated with Con-A irradiated at a high dose.

Conclusions: Our model of chronic food allergy revealed the impact that food irradiation procedures using a low dose has on raising allergenic potential and how irradiation at a high dose might contribute to making allergens innocuous.

Keywords: food allergy; food allergen; food irradiation; Con-A.

Food allergy refers to a group of disorders with an immune response to specific food proteins that affects between 5% of children and 2% of adults (1). Food allergy has been an important and recurring health problem due to the food consumed becoming increasingly processed (2). Protein food allergens are thermostable and resistant to proteases belonging to different groups of plant proteins (3). Lectins, also known as plant agglutinins, are a class of proteins that bind to specific sequences of sugar on cells and glycoproteins (4). Lectins are found in seeds, especially those of legumes. Some lectins are generally recognized as an important anti-nutrient of food (5,6). Concanavalin A (Con-A) is a lectin extracted from jack bean (*Canavalia ensiformis*) extensively characterized and widely applied in biotechnology (7,8).

Current approaches for the management of allergic disease are based on the removal of specific allergens, drug treatments and preventive measures (9). Unfortunately, treatment with medications such as antihistamines and mast cell stabilizers usually play an insignificant role in the treatment of gastrointestinal manifestations (10,11). Genetic engineering offers the opportunity to reduce or even eliminate the compounds in foods that cause allergies (12). Nevertheless, associated concerns about so-called cross-contact allergens cannot be eliminated by the incorporation of genetically modified foods. In addition, preventive measures and elimination of allergens are interdependent and has been a target of regulatory agencies around the world (13).

In this scenario, the development of effective methods to eliminate the antigenic properties of food allergens has been evaluated (14). Among them food irradiation, which is applied to increase the safety and shelf life of foods, has been used (15). Food irradiation can offer a wide range of benefits to the food industry and the consumer. In recent research, we have seen that radiation damages allergens directly by rupturing covalent bonds as a result of transfer of photon energy, or indirectly, by producing reactive oxygen species which can reduce allergenicity by compromising structure-function relationships (16). However, we still need to clarify with the use of other models, if a low dose of radiation attenuates or exacerbates the antigenicity of food allergens when compared to a high dose.

The aim of this study was to establish a chronic allergic with histological changes in mice and to use the model to examine whether a low dose of ionizing radiation is as effective as a high dose to eliminate the antigenicity of food allergens. We monitored

lymphocyte migration in the jejunum and representative cytokines of Con-A-induced allergy as well as the structural and molecular profile of irradiated food allergens.

Methods

Materials

The broad-range molecular weight protein standard, external fluorescence probe 4.4'-bis-1-Anilinonaphthalene 8-sulphonate (bis-ANS) and Concanavalin A from *Canavalia ensiformis* (Jack bean) Type IV were purchased from Sigma Chemical Co., USA. All solvents and other chemicals used were of analytical grade from Merck, Germany. All solutions were made with water purified by the Milli-Q system.

Irradiation of food allergens

Con-A in phosphate buffer (pH 7.2) was lyophilized in borosilicate glass vials (16-125 mm) and then irradiated dry under atmospheric O_2 by a Gammacell 220 Excel 60 Co gamma ray irradiator (Ontario, Canada) using doses of 1.0 and 25.0 kGy at a rate of 8.25 kGy/h. Each dose was analyzed after irradiation by the following methods.

Hemagglutinating activity and protein concentration

Hemagglutinating activity (HA), which was defined as the lowest sample dilution that showed hemagglutination, was evaluated as described by Correia & Coelho (17). Specific HA (SHA) corresponded to the relationship between the HA and protein concentration measured according to Lowry et al. (18) using bovine serum albumin (BSA) as a protein standard in the range of 0-500 µg/mL. The percentage of the remaining SHA ($\%SHA_{REM}$) was calculated according to the equation: $\%SHA_{REM} = (SHA)_{GM} / (SHA)_{GO} \times 100$, where G_M is the Con-A SHA at each radiation dose (1 and 25 kGy) and G_0 is the SHA of non-irradiated Con-A (control).

SDS-PAGE

To detect any insoluble aggregates formed, the precipitate was submitted to gel electrophoresis after centrifugation. SDS-PAGE was performed according to Laemmli (19). Protein samples were resolved on a 10% separating gel and stained with Coomassie blue.

Chromatography analysis

To detect fragmented protein, the supernatant was separated by RP-HPLC after centrifugation. Irradiated samples were submitted to reverse-phase chromatography on a C-4 column (Vydac-Protein Peptide Ultrasphere) performed on an HPLC system (Shimadzu LC-10AD-Tokyo, Japan) and monitored at 280 nm. The column was equilibrated with 0.1% TFA (solvent A) and eluted using 90% acetonitrile/10% H_2O/0.1% TFA (solvent B) in a non-linear gradient, where B = 0% at t = 5 minutes; B = 45% at t = 10 minutes; B = 50% at t = 30 minutes and B = 100% at t = 35 minutes.

Fluorescence and light scattering measurements

Intrinsic fluorescence and light scattering assays were performed on a spectrofluorometer (JASCO FP-6300, Tokyo, Japan). The fluorescence emission intensity of tryptophan from irradiated Con-A was measured in a rectangular quartz cuvette with a 1 cm path-length at 0.1 mg/mL in phosphate buffer, pH 7.2 (25ºC). For intrinsic fluorescence measurements, the excitation was at 295 nm and emission was recorded from 305 to 450 nm, using 5 nm band pass filters for both excitation and emission. For light scattering measurements, the excitation was at 320 nm and emission was recorded from 300 to 340 nm. The light scatted was measured at 90° for the aggregation assays obtained from the area under the fluorescence spectra. The center of spectral mass (CM) was calculated according to Eq. (1): $CM = \sum I_\lambda F_\lambda / \sum F_\lambda$, where F_λ stands for the fluorescence emission at wavelength I_λ and the summation was carried out over the range of appreciable values of F.

Hydrophobic surface analysis

The hydrophobic surface was measured using the same conditions as employed for the intrinsic fluorescence experiment. Samples were transferred to a quartz cuvette and then mixed with 5 μM bis-ANS. The fluorescence emission spectrum was obtained from 400 at 600 nm, with an excitation at 360 nm (20).

Circular dichroism (CD) measurements

CD measurements were carried out on a spectropolarimeter (JASCO J-810, Tokyo, Japan). The instrument was calibrated with D-10-camphorsulfonic acid. The protein concentrations were the following: non-irradiated (5 μM), 1 (5 μM) and 25 kGy (11 μM) in phosphate buffer, pH 7.2. After irradiation, the samples were centrifuged and the measurements were performed with the supernatant. CD spectra were measured in the far-UV range (190-250 nm) in 1 mm path-length quartz cuvette at 25°C. The data were averaged for 8 scans that were performed at a speed of 50 nm/min and collected in 0.5-nm steps. The baselines (buffer alone) were subtracted from the protein spectra. Results were expressed as mean residue ellipticity, (θ), defined as $(\theta) = \theta_{obs}/(10.C.l.n.)$, where θ_{obs} is the CD in millidegrees, C is the protein concentration (M), l is the path-length of the cuvette (cm) and n is the number of amino acid residues assuming a mean number of 237 residues.

Differential scanning calorimetry (DSC) analysis

The thermal stability of native and irradiated Con-A was determined by Differential Scanning Calorimetry (DSC). Thermal analysis was performed using a Microcal VP-DSC micro-calorimeter (Northampton, MA, USA). Prior to all the measurements the buffers and protein solutions were degassed. Thermal transitions were by heating the sample from 5-100°C. A scan rate of 10ºC/h was used. The resulting thermograms were analyzed by the ORIGIN DSC software that was provided by Microcal Inc. (Northampton, USA).

Sensitization and chronic antigen exposure

Female BALB/c mice (5 weeks old) obtained from Laboratório de Imunopatologia Keizo Asami (LIKA) of the Universidade Federal de Pernambuco were maintained under specific pathogen-free conditions and were

given *ad libitum* access to food and water. The animals were kept in an environmentally controlled room, temperature 21 ± 2°C, under a light/dark cycle of 12 hours. Requirements for care and handling of experimental animals were according to international and Brazilian regulations. All test substances were administered intragastrically by tube. Con-A was dissolved in 0.5 mL of 0.9% sterile saline.

BALB/c mice were immunized subcutaneously on day 0; 15 and 30 using 0.5 ml Con-A (10 µg/mL) dissolved in saline without use of an adjuvant (eight mice per group). Control animals were treated subcutaneously with 0.5 mL saline. Three days before starting the oral treatment in animals, they were stimulated with the same dose intraperitoneally. During 28 days, mice were treated as follows: group A, immunized mice were treated with 1 mL saline/day; group B, immunized mice were treated with non-irradiated Con-A; group C and D immunized mice were treated with irradiated Con-A at 1 and 25 kGy, respectively. The dose of bean lectin (27 mg/kg body weight/day) was according to total dietary intake of lectins in human subjects consuming vegetarian diets - calculations based on data from Peumans and van Damme (21).

Body weight and leukocyte evaluation

Body weight was determined before and after immunization and after oral challenge. The final body weight of each group was obtained from the means of the individual values and expressed in grams (g). Blood samples were obtained and placed into micro-blood tubes containing the anticoagulant ethylenediamine tetra-acetic acid (EDTA). Hematological indices were determined by an automated particle counter, random-access clinical hematologic analyzer Coulter STK-S (Hospital das Clínicas, UFPE, Recife, Brazil). Hematological indices were confirmed by optical microscopy, in which the morphology of leukocytes stained by the Giemsa method was observed.

Multiplex cytokine analysis

Multiple cytokine analysis kits were obtained by Genese Produtos Diagnósticos Ltda (São Paulo, SP, Brazil). Millipore multiscreen, 96-well filter plates (Bedford, MA, USA) were used for all multiplex cytokine kits. Assays were run in triplicate according to the manufacturers' protocols. Data were collected using the Milliplex Analyser 200 version 2.3 (Luminex, Austin, USA). Data analysis was performed using the Analyst software, version 3.1. A four-parameter regression formula was used to calculate the sample concentrations from the standard curves.

Histology

After 28 days of oral challenge, the mice were sacrificed by decapitation. The histopathological evaluation of organs (jejunum) of animals was performed with an optical microscope. After mounting, the preparations were evaluated using a video-microscopy system (MOTIC BA200 Microscope, digital camera with Motic 1000-1.3M Pixel USB 2.0).

Statistical analysis

Differences between treatment groups of *in vivo* experiments were performed using nonparametric test (Kruskal-Wallis test) followed by the *post hoc* Dunns test in GraphPrism® (GraphPad Software Inc., San Diego, CA, USA). Statistical significance was established at $p < 0.05$.

Results and Discussion

Structural analysis of food allergen after irradiation

Low doses of ionizing radiation (up to 1 kGy) are usually applied to control food-borne pathogens, reduce microbial load and insect infestation, inhibit the germination of root crops, and extend the shelf-life of perishable produce for commercial purposes (22). It is possible, however, that the types of modification that food proteins may undergo during irradiation include protein unfolding and aggregation, may not be observed at a low-dose range. Thus, the potential to affect structural stability, and hence the form in which allergens are presented to the immune system after a low dose of radiation compared with a high dose was investigated. The SHA of Con-A in its irradiated forms revealed major changes in activity at a high dose (Fig. 1a). At 1 kGy, the SHA was reduced by around 20%. The change in structural basis for selective sugar recognition and binding was affected at a high dose. Irradiation inhibits cell agglutination (16) which may affect allergen binding to the gut and reduce the allergic effect.

In order to evaluate alteration in the structure of Con-A after irradiation, the samples were analyzed by SDS-PAGE. Non-irradiated Con-A showed protein bands with 25, 13 and 11 kDa. As shown by Wang et al. (23), N-terminal sequence determination of the lighter components indicated that they corresponded to the N-terminal portion (13 kDa) and the carboxyl terminal portion (11 kDa) of the intact protein. SDS-PAGE profiles show that a high dose of γ-radiation induces peptide bond cleavages which later undergo aggregation (Fig. 1b). At low-dose, even in the denaturing conditions of SDS-PAGE, fragmentation and molecular aggregation were not observed. Non-irradiated Con-A was separated by RP-HPLC into three components, A1, A2 and A3 (intact protein). The reverse phase chromatography analysis revealed a profound change in the elution profile at 25 kGy, as indicated in Fig. 1c. No significant change was detected to Con-A after exposure to a low dose. As shown in Fig. 1d, the scattering spectra of irradiated Con-A showed a shape

characteristic of proteins with a high degree of aggregation. This aggregation frequently occurs through inter-protein cross-linking reactions, and hydrophobic and electrostatic interactions after irradiation (24,25).

The phenomenon of the conformational stability of irradiated versus non-irradiated Con-A was investigated by fluorescence spectroscopy, circular dichroism and DSC. Since the intensity of the intrinsic fluorescence spectra of proteins is easily affected by many environmental factors, the center of spectral mass (CM) was used to reflect the structural integrity of the protein. The typical spectra are presented in Fig 2a. After irradiation at 1 kGy, no significant change was observed in the CM value. For samples irradiated at 25 kGy, the intrinsic fluorescence spectra had a red-shift of about 8 nm. These observations suggested that the inactivation of Con-A at a high dose was caused by irreversible changes in Con-A tertiary structure. Bis-ANS fluorescence was weakly observed in the non-irradiated Con-A. After high-dose irradiation, bis-ANS fluorescence increased, with its peak shifted to 497 nm and its intensity increased (Fig. 2b).

The CD spectrum recorded at 25°C of non-irradiated Con-A exhibited a typical shape of β proteins (Fig 3a). At a high dose, the shape of the CD spectra revealed that irradiated protein was dominated by a lack of β-sheet structures. This was reflected by the lack of single negative peak at around 222 nm. To elucidate the profile of non-native oligomers that still have β-sheet structures after low-dose irradiation, differential scanning calorimetry (DSC) experiments were performed. As shown in Fig. 3b, the heat flow versus temperature profile showed only one endothermic peak, suggesting that the thermal denaturation of non-irradiated Con-A, a tetrameric protein at pH 7.2; follows the dissociative mechanism of denaturation as $2N2 \leftrightarrow D \rightarrow U$ (26). The main peak was centered at about 63°C. For Con-A irradiated at 1 kGy, the main peak was centered at about 57°C with a pre-transition at around 37°C. For samples irradiated at 25 kGy, the DSC was not run due to higher protein aggregation.

The occurrence of red-shift in the intrinsic fluorescence spectra and lack of native β-rich secondary structures in irradiated Con-A, indicates the accumulation of completely unfolded, fragmented peptides that strongly bind to bis-ANS and have a tendency to form insoluble amorphous aggregates after a high dose. The formation of aggregates is important because they can eventually be removed during food processing with a lower level of

allergens and, therefore, a lower allergenic property. However, what is the real effect of aggregation on managing allergen risks? And if there is a risk, what kind of reaction is triggered? To this end, we decided to evaluate the effect of food irradiation on food allergens using a chronic food allergy model.

Treatment schedule

Currently, little is known about how irradiation may alter food allergens, and hence there is a need to systematically investigate the relationship between food protein allergenicity and the effect of food irradiation. However, we know that the decrease or increase of allergenicity can be caused by protein unfolding, mis-folding as well as by aggregation. Sanchez and Frémont (27) have extensively reviewed the effects of food processing on the structural stability of food allergens and concluded that effects of protein–protein interactions (especially aggregation) are virtually unknown in attempting to reduce/eliminate food allergens. To investigate these effects, we describe a BALB/c mice model of chronic food allergy that were given non-irradiated and irradiated Con-A. We monitored morphological changes and lymphocyte infiltration in the small intestine as well as body weight, blood leukocytes, cytokine secretions of IL-4, IL-5, eotaxin and rantes.

Table 1 shows the significant weight loss ($p < 0.05$) for non-irradiated and irradiated Con-A at 1 kGy of BALB/c mice after oral administration by 28 days. However, despite a significant difference ($p < 0.05$) for non-irradiated and control groups, there was no weight loss after sensitization to mice treated with Con-A irradiated at 25 kGy. The analysis of cytokines and chemokines revealed a significant increase ($p < 0.05$) for eotaxin but not significant for IL-4 and IL-5 in mice treated with non-irradiated con-A when compared to the control group (Table 1). To our surprise, mice treated with Con-A irradiated at 1 kGy showed a significant increase ($p < 0.05$) in levels of eotaxin and IL-4 compared to animals treated with non-irradiated Con-A. While mice treated with Con-A irradiated at 25 kGy showed normal levels of cytokines compared to control animals.

The treatment of sensitized animals subjected to intragastric administration of non-irradiated Con-A showed a lymphocytic infiltrate in the stroma of the microvilli and in the colon zone, where the crypts and the villi are mixed, when compared with untreated immunized mice (Fig. 4 a-b). High levels of leukocyte infiltration with eosinophils can also be found (Fig. 4 c-d). However, the jejunum from immunized animals treated with

irradiated Con-A (1 kGy) showed a lymphocytic infiltrate filling completely the stroma of microvilli without a real definition of the tubular glands and submucosa (Fig. 5 a-b). On the other hand, in immunized animals treated with irradiated Con-A (25 kGy), infiltration with less intensity was observed (Fig. 5 c-d).

Classic findings of food allergy were observed in mice treated with native Con-A (non-irradiated). The weight loss, increase in cytokines IL-4, IL-5, eotaxin and leukocyte infiltration after administration on gut has been previously observed (28-30). Food processing may help inactivate certain conformational epitopes on some, but not all, allergens and is unlikely to eliminate linear epitopes (14). Food irradiation can be effective in eliminating allergens after a high dose, since fragmentation of the molecular structure was observed.

To our knowledge, this is the first direct demonstration of effects from irradiated allergens on a chronic food allergy model. The development of this phenotype shows lymphocytic infiltration, it also leads to the persistence of silent inflammation within the gut. Moreover, all phenotypes mapped in this study were not expressed in allergens irradiated at high-dose. On the other hand, a low dose of radiation exacerbates the phenotype and can cause severe allergic inflammatory responses. This effect may be due to increased exposure of conformational and linear epitopes resulting from the formation of partially unfolded and aggregated species after irradiation. Further investigations into the impact of food irradiation on allergen structure and allergenic potential are still warranted. However, two areas that had been previously neglected were clarified in our study: 1) impact that food processing procedures have on allergenic potential 2) the way in which food irradiation may alter allergic reactions in sensitized animals. Yet, the exact mechanism, including interactions between chemokines and cytokines as well as conformational and linear epitope modifications, remains to be further investigated.

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Figure captions

Fig. 1 - Effect of γ-radiation on Con-A structure. (**a**) The percentage of remaining specific hemagglutination activity, $\%SHA_{REM}$. (**b**) SDS–PAGE profile from irradiated Con-A.

(MW) Molecular weight; (C) non-irradiated; (1S) precipitate 1kGy; (1P) soluble 1kGy; (25S) precipitate 25kGy; (25P) soluble 25kGy. Arrows indicate high-molecular-weight molecules. (**c**) Reverse phase chromatography in an HPLC system. (—) Non-irradiated and irradiated Con-A at (—) 1 and (—) 25 kGy. (**d**) Light scattering for the aggregation assays; excitation (320 nm) and emission (300–340 nm).

Fig. 2 - Intrinsic and bis-ANS fluorescence. (**a**) Center of spectral mass of intrinsic fluorescence; Lectin excitation at 295 nm and emission at 305-450 nm. (**b**) Center of spectral mass of bis-ANS fluorescence; excitation at 360 nm and emission at 400-600 nm.

Fig. 3 - Far-UV CD spectra and DSC thermograms. (**A**) CD spectra were measured in the far-UV range (190-250 nm) in 1 mm path-length quartz cuvette. (θ) is given in degree squared centimeters per decimole. (■) Non-irradiated and irradiated Con-A at (Δ) 1 and (○) 25 kGy. (**B**) Con-A thermal denaturation monitored by DSC. The heating rate was 10ºC/hour. The protein concentration was 1 mg/mL. (—) Non-irradiated and irradiated Con-A at (—) 1 kGy.

Fig. 4 - Photomicrograph of jejunal mucosa of untreated mice and mice treated with native Con-A. (**a**) Jejunum of immunized animals treated with saline: Note the lymphocytic infiltrate filling the stroma of microvilli (mv) and also around the tubular glands or crypts (cr). (**b**) High numbers of enterocytes (e) with a prominent striated cuticle. Central stroma with evident lymphocytic infiltration. (**c**) Jejunum of animal treated with non-irradiated Con-A: there is lymphocytic infiltrate in the stroma of the microvilli (mv) and in the colon zone (cz), where the crypts and the villi are mixed. (**d**) Leukocyte infiltration with numerous eosinophils (eo) in animals treated with non-irradiated Con-A.

Fig. 5 - Photomicrograph of jejunal mucosa of animals treated with irradiated Con-A. (**a**) Jejunum of animals treated with Con-A irradiated at a low dose: lymphocytic infiltrate is observed filling completely the stroma of microvilli (mv) and no definition of tubular glands (tb) and submucosa (sm) can be seen because the same infiltration (**b**). (**c**) Jejunum of animals treated with Con-A irradiated at a high dose where the same infiltration already mentioned for the 1 kGy dose can be observed but with less intensity (**d**).

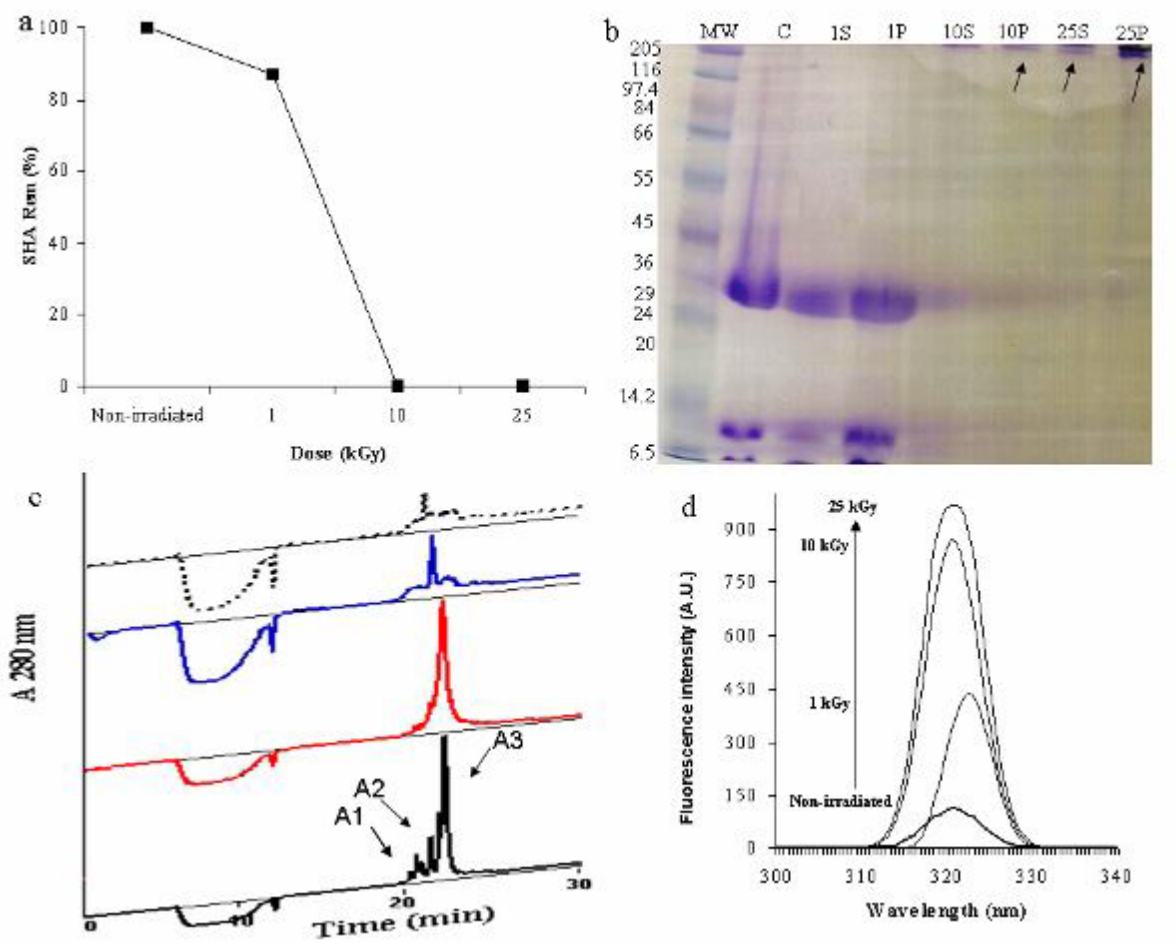

a
SHA Rem (%)
Non-irradiated
1
10
25
Dose (kGy)
b
MW
C
1S
1P
10S
10P
25S
25P
205
116
97.4
84
66
55
45
36
29
24
20
14.2
6.5
c
A 280 nm
A1
A2
A3
Time (min)
d
Fluorescence intensity (A.U.)
25 kGy
10 kGy
1 kGy
Non-irradiated
Wavelength (nm)

Figure 1

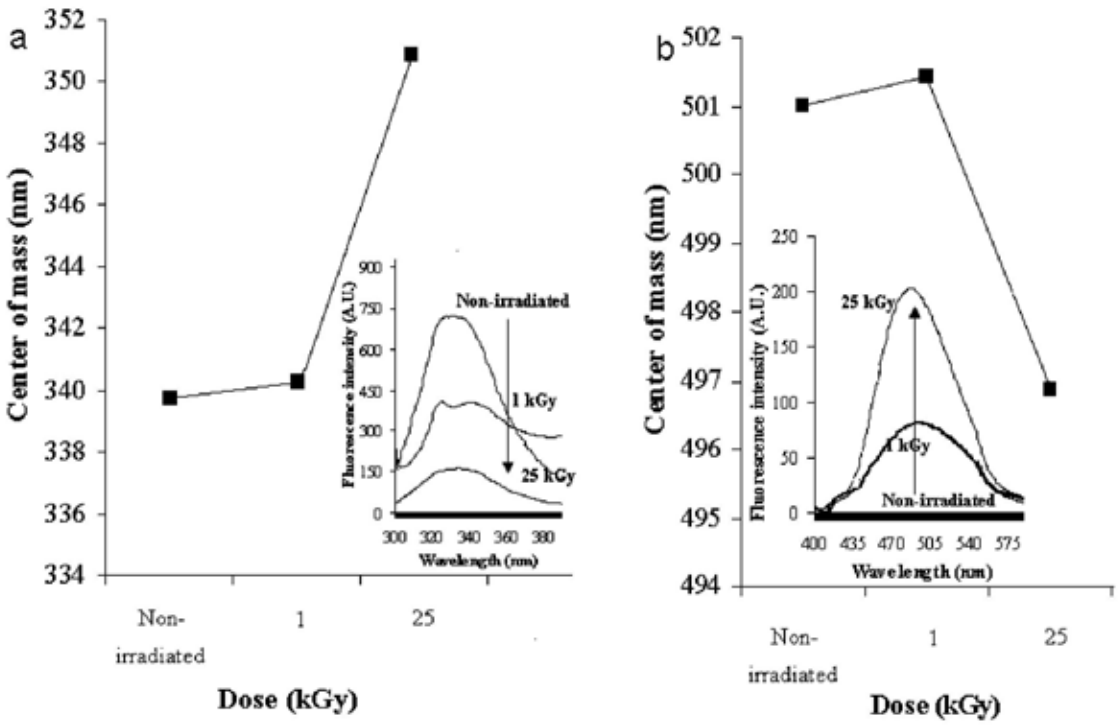

a
Center of mass (nm)
352
350
348
346
344
342
340
338
336
334
Non-irradiated
1
25
Dose (kGy)
Fluorescence intensity (A.U.)
900
750
600
450
300
150
0
300 320 340 360 380
Wavelength (nm)
Non-irradiated
1 kGy
25 kGy
b
Center of mass (nm)
502
501
500
499
498
497
496
495
494
Non-irradiated
1
25
Dose (kGy)
Fluorescence intensity (A.U.)
250
200
150
100
50
0
400 435 470 505 540 575
Wavelength (nm)
25 kGy
1 kGy
Non-irradiated

Figure 2

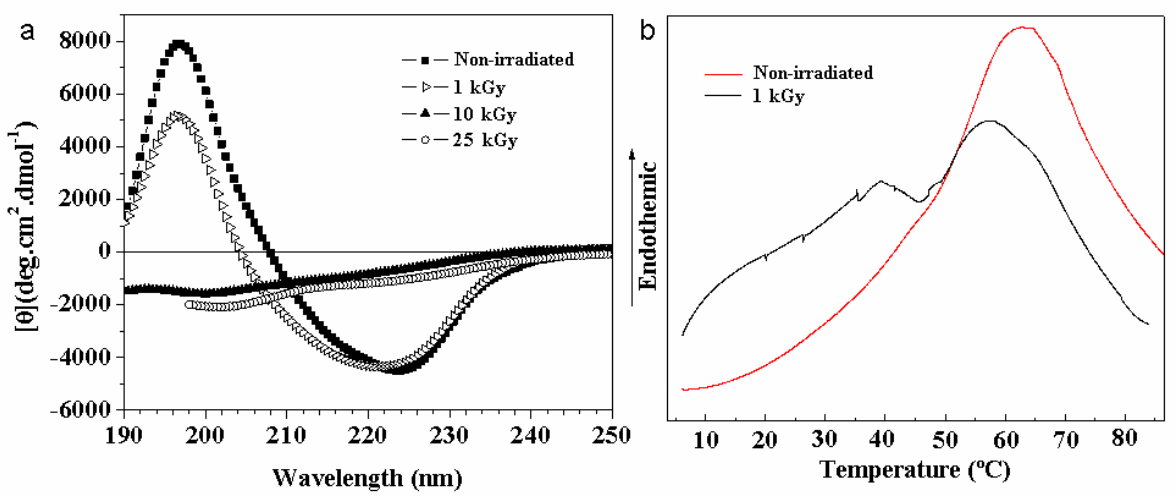

a
8000
6000
4000
2000
0
-2000
-4000
-6000
190
200
210
220
230
240
250
[θ](deg.cm^2.dmol^{-1})
Wavelength (nm)
Non-irradiated
1 kGy
10 kGy
25 kGy
b
Non-irradiated
1 kGy
Endothemic
10
20
30
40
50
60
70
80
Temperature (ºC)

Figure 3

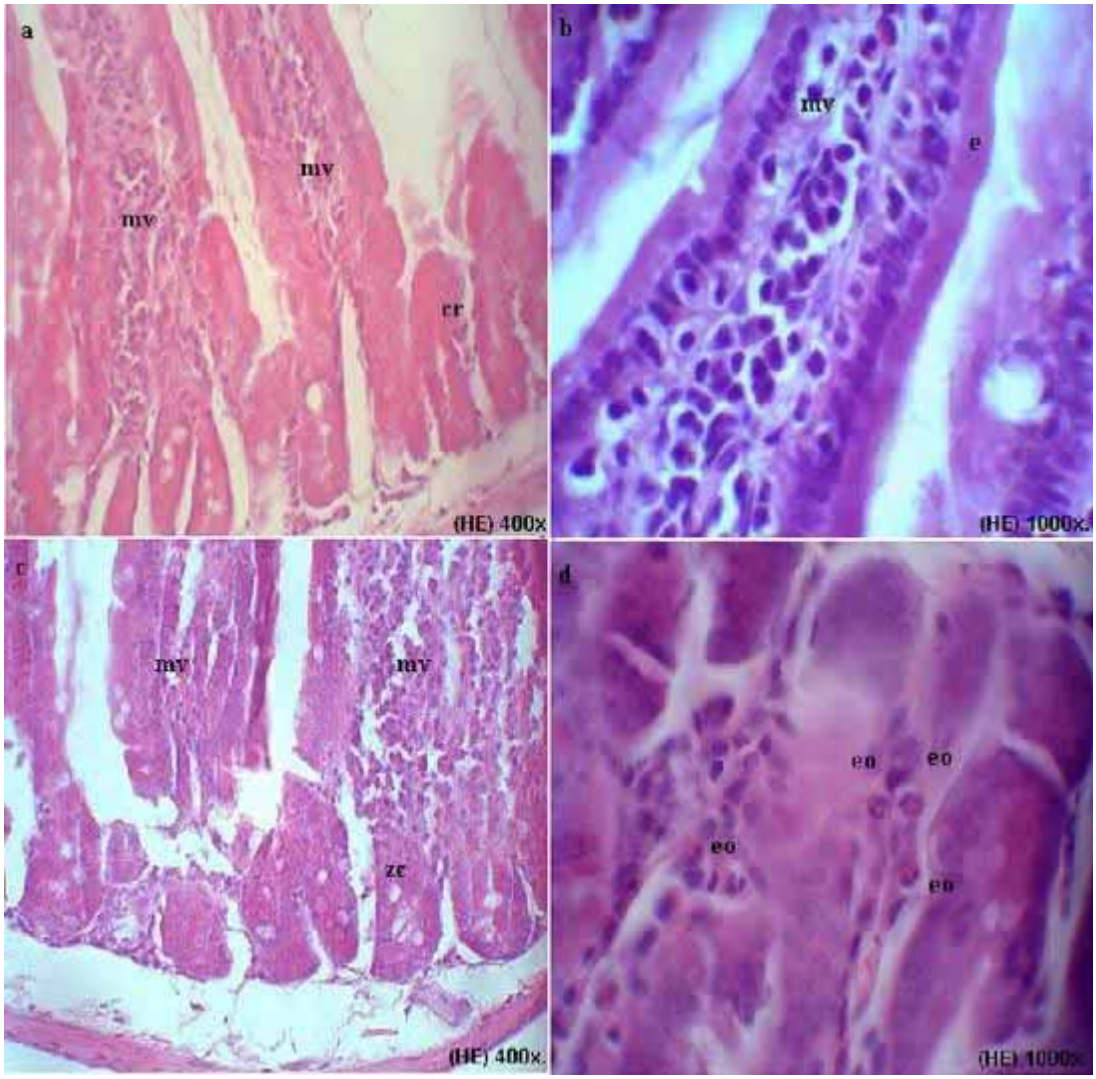
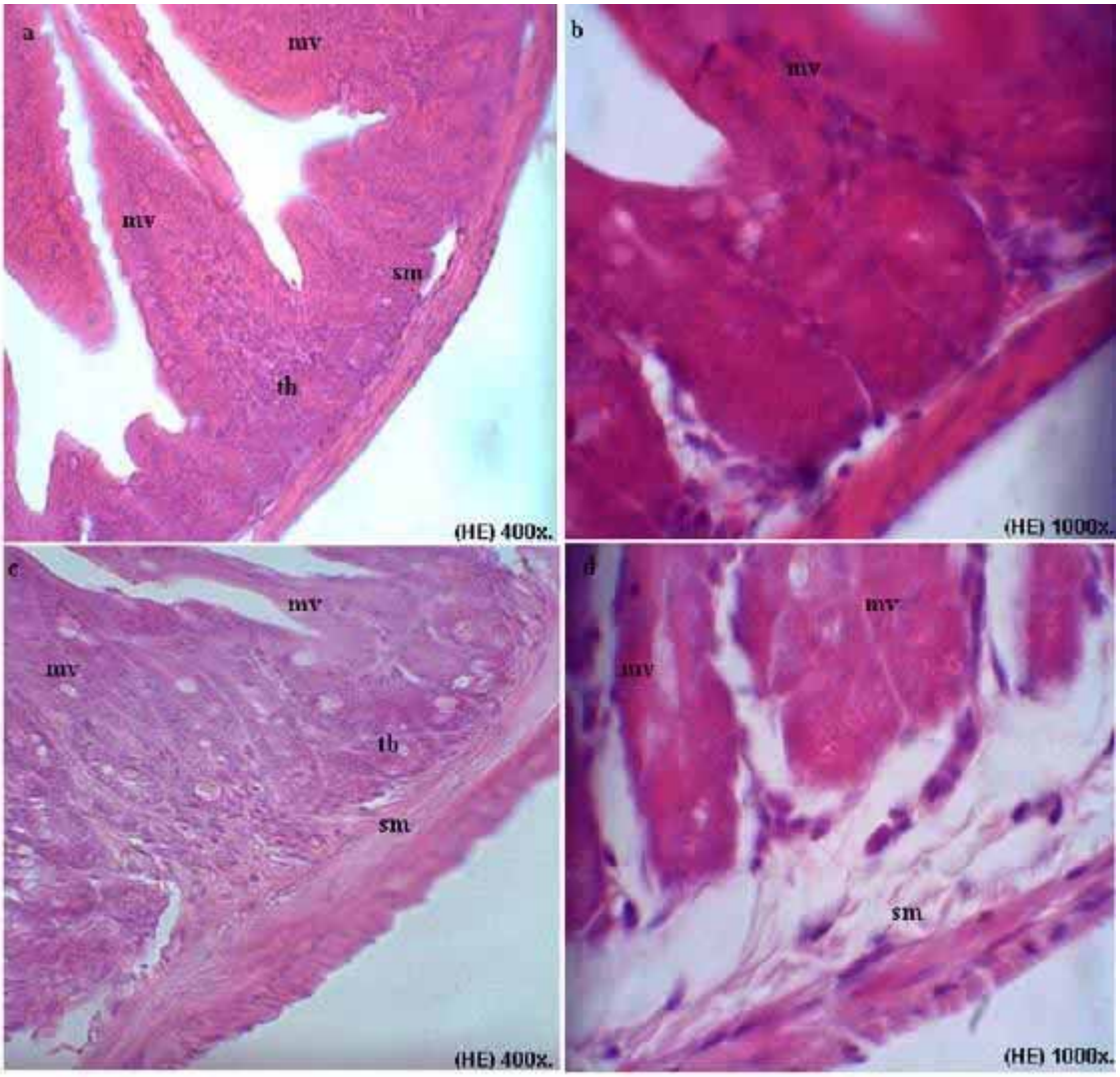

a
mv
mv
cr
(HE) 400x
b
mv
e
(HE) 1000x
c
mv
mv
zc
(HE) 400x
d
eo
eo
eo
eo
(HE) 1000x

Figure 4


a
mv
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(HE) 400x.
b
mv
(HE) 1000x.
c
mv
mv
th
sm
(HE) 400x.
d
mv
mv
sm
(HE) 1000x.

Figure 5

Table 1 - Body weight, leukocytes and cytokine profiles of chronic allergic mice model.

	Groups			
Weight (g)	**Go**	**G1**	**G2**	**G3**
Initial	34.7 ± 1.0	28.1 ± 2.1	28.8 ± 1.1	27.6 ± 0.3
Post immunization	41.0 ± 0.8	31.1 ± 3.2*	32.2 ± 1.7*#	36.3 ± 0.7*#
Post treatment	43.1 ± 1.0	27.6 ± 3.7*	29.6 ± 2.3*#	36.4 ± 0.7*#
White blood count				
WBC[a]	1.5 ± 0.3	4.2 ± 2	4.0 ± 1.1	3.8 ± 0.4
Large lymphocytes[b]	80 ± 4.8	91.7 ± 1.0*	80.8 ± 16.4	86.2 ± 4.4
monocytes[b]	4.2 ± 2.7	1.9 ± 0.4	2.3 ± 1.4	1.4 ± 0.5
Neurophils[b]	13.8 ± 3.2	3.5 ± 0.8*	12.6 ± 12.8	7.9 ± 3.7
Eosinophila[b]	1.8 ± 1.0	2.9 ± 1.1	4.1 ± 3.5	0.5 ± 0.2
Cytokines and chemokines				
Eotaxin (pg/mL)	435.4 ± 37.6	1086.2 ± 61.8*	1438.6 ± 776.5*	595.5 ± 116.2*
IL-4 (pg/mL)	< 0.31	15.3 ± 2.8	27.5 ± 17.1*	16.7 ± 9.4
IL-5 (pg/mL)	15.9 ± 2.8	211.6 ± 63.8	21.3 ± 9.6#	17.2 ± 15.9#
Rantes (pg/mL)	41.4 ± 3.1	76.1 ± 15	67.8 ± 27.9	26.9 ± 10.0#$

Group Go, immunized control animals; group G1, immunized mice were treated with non-irradiated Con-A; group G2 and G3 immunized mice were treated with irradiated Con-A at 1 and 25 kGy, respectively. [a]White blood count in thousand per cubic millimeter. [b]Mean per cent of total leukocytes. * $p < 0.05$ compared to immunized control animals; # $p < 0.05$ compared to immunized mice treated with non-irradiated Con-A; $ $p < 0.05$ compared to immunized mice treated with irradiated Con-A at low-dose.

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Molecular fragmentation of wheat-germ agglutinin induced by food irradiation reduces its allergenicity in sensitised mice

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ABSTRACT

WGA, an agglutinin from wheat germ which is largely responsible for many of wheat's allergies, was used as a model to investigate the action of ionising radiation on WGA's anti-nutritive effects in sensitised mice. Based on the molecular structure, the present study also examined the structural modification of WGA in relation to the range of dose. Structural integrity was monitored using HPLC, fluorescence spectrometry and circular dichroism. Results showed a loss of intrinsic activity and the formation of insoluble amorphous aggregates with a lack of native conformational structures after irradiation. Current findings suggest that the allergenic epitopes of WGA became less active and antigenic after high-dose radiation. The reduction of cytokines typical of allergic reactions, with decreased lymphocytic infiltrate, was observed in the gut of mice given irradiated versus native WGA. Food irradiation proved effective and safe in combating immunological and allergic effects of WGA.

1. Introduction

Lectins are proteins or glycoproteins which are widely distributed in crop plants and which have the ability to bind to cellular surfaces by means of specific glycol-conjugates (Breiteneder & Ebner, 2000). Because of this binding property, lectins are recognised as the major anti-nutrient of food. When consumed in excess by sensitive individuals, they can cause three primary physiological reactions: they can cause severe intestinal damage disrupting digestion; they can provoke IgG and IgM antibodies causing food allergies; and they can bind to erythrocytes causing haemagglutination and anaemia (Hamid & Masood, 2009). The anti-nutritional effects of lectins found in cereal grains are similar because they are closely related to one another both structurally and immunologically (Peumans & Cammue, 1986).

In wheat, gliadin, a component of gluten and the isolectin of wheat germ agglutinin (WGA), is involved in almost every acute and chronic inflammatory disorder, including neurodegenerative disease, inflammatory bowel disease, infectious and autoimmune diseases (Jones & David, 2005). WGA, the minor allergen from wheat, is a heat-stable protein and resistant to digestive proteolytic breakdown, which results in increased endogenous nitrogen losses and depressed growth rate in young animals (Cordain, 1999). In cereals, WGA concentrations range from 13 to 53 mg/kg. The highest WGA concentrations are found in wheat germ. The estimated quantity of total dietary lectin is in the range 0 ± 200 mg/person per day (Watzl, Neudecker, Haĕnsch, Rechkemmer, & Pool-Zobel, 2001).

Linear epitopes are a series of adjacent amino acids with no requirement for a particular secondary or tertiary structure, whereas a conformational epitope is strictly dependent on the folding of the protein chain (Restani et al., 2004). Because of their resistance to proteolytic breakdown, food allergens may facilitate the passage of undegraded conformational and linear epitopes into the systemic circulation, by their ability to increase the permeability of the intestine (Sjolander, Magnusson, & Latkovic, 1984). Therefore, compact three-dimensional structure, disulphide bonds and glycosylation, which may contribute to protein stability, are significant factors for the resistance of food allergens to routine food processing (Breiteneder & Mills, 2005a).

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Protein denaturation which involves structural or conformational changes to the native structure without alteration of the amino acid sequence has been obtained by various food processing techniques (Sathe, Teuber, & Roux, 2005). In these circumstances, however, only conformational epitopes are severely affected. Nevertheless, recent research has revealed the potential use of food irradiation as an alternative tool in reducing the antigenic characteristics of food allergens. The complete abolition of intrinsic activity and of molecular structure with the formation of insoluble amorphous aggregates has been a common finding after high doses of gamma irradiation (Vaz et al., 2011).

The safety and benefits of foods processed by ionising radiation are well recognised and over 30 countries use it for commercial purposes (Kume, Furuta, Todoriki, Uenoyama, & Kobayashi, 2009). However, our lack of knowledge of how a processed allergen can cause a problem, and of the analytical methodology with appropriate performance to trace it, is a reality. Thus, it is critically important to investigate the relationship between allergenicity and the stability of allergens after food processing, in order to help control the apparently rising tide of food allergies. Consequently, as a first step towards this, the main aim of the present work was to examine the effects of γ-radiation over a broad dose range on the molecular structure of WGA. We also investigated the degree of cellular infiltration in the gut and representative cytokines of Swiss albino mice sensitised and subjected to oral doses with irradiated WGA for 7 days when compared to non-irradiated WGA.

2. Materials and methods

2.1. Chemicals

The external fluorescence probe, 4,4′-bis-1-anilinonaphthalene 8-sulphonate (bis-ANS), and WGA (catalogue number L9640, highly purified) from *Triticum vulgaris* (wheat) were purchased from Sigma Chemical Co., USA. All solvents and other chemicals used were of analytical grade from Merck, Darmstadt, Germany. All solutions were made with water purified by the Milli-Q system.

2.2. WGA irradiation

WGA in phosphate buffer (pH 7.2) was lyophilised in borosilicate glass vials (16–125 mm) and then irradiated dry under an O_2 atmosphere by a Gammacell 220 Excel ^{60}Co gamma ray irradiator (Ottawa, Ontario, Canada) using doses of 1.0, 10.0 and 25.0 kGy at a rate of 8.25 kGy/h. Each dose was analysed after irradiation by the following methods.

2.3. Inactivation and unfolding

Haemagglutinating activity (HA), which was defined as the lowest sample dilution that showed haemagglutination, was evaluated as described by Correia and Coelho (1995). Specific HA (SHA) corresponded to the relationship between the HA and protein concentration measured according to Lowry, Rosebrough, Farr, and Randall (1951), using bovine serum albumin (BSA) as a protein standard in the range of 0–500 μg/mL. The percentage of the remaining SHA (%SHA_{REM}) was calculated according to the equation: $\%SHA_{REM} = (SHA)_{G_M}/(SHA)_{G_0} \times 100$, where G_M is the WGA SHA at each radiation dose (1, 10 and 25 kGy) and G_0 is the SHA of non-irradiated WGA (control).

To detect the nature of insoluble aggregates, the precipitate was treated with a chaotropic agent (8 M urea) and analysed by RP-HPLC after sample centrifugation. Irradiated samples were submitted to reverse-phase chromatography on a C-4 column (Vydac-Protein Peptide Ultrasphere – 4.6 × 150 mm, 5 μm particle size, 300 Å pore size) performed on an HPLC system (Shimadzu LC-10AD; Kyoto, Japan) and monitored at 215 nm. The column was equilibrated with 0.1% TFA in water (solvent **A**) and eluted using 90% acetonitrile/10% H_2O/0.1% TFA (solvent **B**) in a non-linear gradient, where **B** = 0% at t = 5 min; **B** = 45% at t = 10 min; **B** = 50% at t = 30 min and **B** = 100% at t = 35 min.

Unfolding and aggregation of WGA was monitored by intrinsic fluorescence and light scattering using a spectrofluorometer (JASCO FP-6300, Tokyo, Japan). A protein concentration of 0.150 mg/mL, in 100 mM sodium phosphate buffer (pH 7.2) was used. The fluorescence emission intensity of tryptophan from irradiated WGA solution was measured at 25 °C in a rectangular quartz cuvette with a 1-cm path length. For intrinsic fluorescence measurements, the excitation was at 295 nm and emission was recorded from 305 to 450 nm, using 5-nm band pass filters for both excitation and emission. For light scattering measurements, the excitation was at 320 nm and emission was recorded from 300 to 340 nm. The light scattering was measured at 90° for the aggregation assays, obtained from the area under the fluorescence spectra. The hydrophobic surface was measured using the same conditions as employed for the intrinsic fluorescence experiment. Samples were transferred to a quartz cuvette and then mixed with 5 μM bis-ANS. The fluorescence emission spectrum was obtained from 400 at 600 nm, with an excitation at 360 nm (Bhattacharyya, Mandal, Banerjee, & Roy, 2000). The centre of spectral mass (CM) was calculated according to Eq. (1):

$$CM = \sum I_i F_i / \sum F_i, \quad (1)$$

where F_i stands for the fluorescence emission at wavelength I_i and the summation was carried out over the range of appreciable values of F.

Far-UV CD spectra were recorded in the 190–250 nm region, in a 1-mm path length quartz cuvette using a spectropolarimeter (JASCO J-810). The instrument was calibrated with D-10-camphorsulfonic acid. The protein concentrations were as follows: non-irradiated (8 μM), 1 (8 μM), 10 (10 μM) and 25 kGy (20 μM) for WGA, in phosphate buffer, pH 7.2 at 25 °C. After irradiation the samples were centrifuged and the measurements were performed with the supernatant. The data were averaged for eight scans that were performed at a speed of 50 nm/min and collected in 0.5-nm steps. The baselines (buffer alone) were subtracted from the protein spectra. Results were expressed as mean residue ellipticity, $[\theta]$, defined as

$$[\theta] = \theta_{obs}/(10.C.l.n),$$

where θ_{obs} is the CD in millidegrees, C is the protein concentration (M), l is the path-length of the cuvette (cm) and n is the number of amino acid residues assuming a mean number of 186 residues.

2.4. Sensitisation and oral challenge

Female Swiss albino mice (5 weeks old) were obtained from the breeding colony of the Departamento de Antibióticos da Universidade Federal de Pernambuco, Brazil, and given *ad libitum* access to food and water. The animals were kept in an environmentally controlled room, temperature 21 ± 2 °C, under a light/dark cycle of 12 h. Requirements for care and handling of experimental animals were according to international and Brazilian regulations. All test substances were administered intragastrically by tube. WGA was dissolved in 0.5 mL of 0.9% sterile saline.

Swiss albino mice were immunised subcutaneously on Day 0, 15 and 30, using 0.5 ml WGA (10 μg/mL) dissolved in saline without use of an adjuvant (five mice per group). Control animals were treated subcutaneously with 0.5 mL saline. Three days before starting the oral treatment in animals, they were stimulated with the same dose intraperitoneally. Over 7 days, mice were treated thus: group A, immunised mice were treated with 1 mL saline/day; group B,

immunised mice were treated with non-irradiated WGA; group C and D immunised mice were treated with irradiated WGA at 1 and 10 kGy, respectively. The dose of WGA (27 mg/kg body weight/day) was according to total dietary intake of lectins in human subjects consuming vegetarian diets (calculations based on data from Peumans & Van Damme, 1996).

2.5. Body weight and leukocytes evaluation

Body weight was determined before and after immunisation and after oral challenge. The final body weight of each group was obtained from the means of the individual values and expressed in grams. Blood samples were obtained and placed into micro-blood tubes containing the anticoagulant ethylenediaminetetra-acetic acid (EDTA). Haematological indices were determined by an automated particle counter, random-access clinical haemato-logic analyser Coulter STK-S (Hospital das Clínicas, UFPE, Recife, Brazil). Haematological indices were confirmed by optical micros-copy, in which the morphology of leucocytes stained by the Giemsa method was observed.

2.6. Multiplex cytokine analysis

Multiplex cytokine analysis kits for mice were obtained by Genese Produtos Diagnósticos Ltda (São Paulo, SP, Brazil). Millipore multiscreen 96-well filter plates (Bedford, MA) were used for all multiplex cytokine kits. Assays were run in triplicate according to the manufacturers' protocol. Data were collected using the Milliplex Analyser 200 version 2.3 (Luminex, Austin, USA). Data analysis was performed using the software Analyst version 3.1. A four-parameter regression formula was used to calculate the sample concentrations from the standard curves.

2.7. Histology

After 7 days of oral challenge, the mice were sacrificed by decapitation. The histopathological evaluation of organs (jejunum) of animals was performed with an optical microscope. Fragments of organs were fixed in formalin (10%) and were subsequently dehydrated in a series of alcohols (70–100%), cleared in xylene and embedded in paraffin. Histological sections of 5 μm were

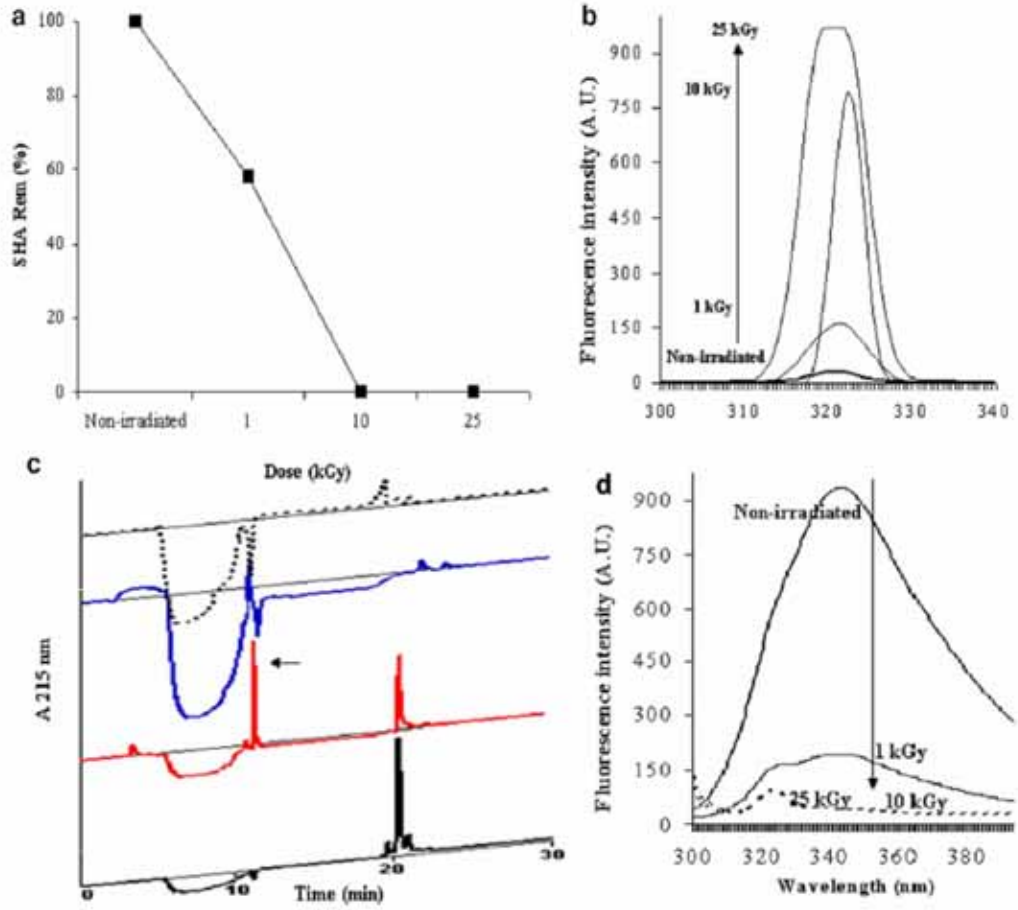

Fig. 1. Effect of γ-radiation on WGA. (a) The percentage of remaining specific haemagglutination activity, %SHA_{Rem}. (b) Light scattering for the aggregation assays; excitation (320 nm) and emission (300–340 nm). (c) Reverse phase chromatography by HPLC. (–) Non-irradiated and irradiated WGA at (—) 1; (—) 10 and (. . .) 25 kGy. (d) Intrinsic fluorescence; excitation at 295 nm and emission at 305–450 nm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

stained by routine histologic haematoxylin and eosin (HE) and mounted between slide and coverslip with synthetic resin. After mounting, the preparations were evaluated using a video-microscopy system (MOTIC BA200 Microscope, digital camera with Motic 1000–1.3 M Pixel USB 2.0).

2.8. Statistical analysis

Differences between treatment groups of *in vivo* experiments were performed using nonparametric test (Kruskal–Wallis test) followed by the *post hoc* Dunns test in GraphPrism® (GraphPad Software Inc., San Diego, CA). Statistical significance was established at $p < 0.05$.

3. Results and discussion

We have previously shown that food irradiation induces a loss of intrinsic activity in food allergens (Vaz et al., 2011). This loss was completely dependent on the structural change elicited by molecular fragmentation, because the primary structure from irradiated allergens has been affected. For WGA, the change in the activity profile at each range dose is demonstrated in Fig. 1a. WGA is a homodimeric protein containing 16 disulphide bridges. The monomers associate with each other in a head-to-tail fashion forming a twofold symmetric globule. Each of the four carbohydrate-binding sites of WGA is located at the interface of two intercatenary domains (Muraki, Ishimura, & Harata, 2002). According to Pusztai et al. (1993), it is particularly worrying that detectable amounts of functionally-intact WGA are transported across the intestinal wall and may reach the systemic circulation, due to the heat stability and its resistance to proteolytic breakdown. However, as estimated, irradiation inhibited cell agglutination *via* WGA, which may affect its binding to the gut and reduce the allergic effect.

Since WGA exhibited a change in the activity profile due to intense aggregation, as detected by light scattering (Fig. 1b), the aggregate was treated with chaotropic agent (8 M urea) and analysed by RP-HPLC after sample centrifugation. Samples showed dissociation of the aggregates, suggesting that irradiation may induce peptide bond cleavage as well as complete fragmentation of the polypeptide chains at doses above 10 kGy. Non-irradiated WGA was separated by RP-HPLC into only one peak. The chromatography analysis revealed changes after exposure at 1 kGy, as indicated in Fig. 1c. The appearance of a previous peak (Fig. 1c – arrow) to the main peak indicates partial fragmentation of the WGA at this dose.

Conformational stability of WGA was investigated using fluorescence and CD spectroscopy. The shift in tryptophan fluorescence intensity and ellipticity at ~225 nm were observed with increasing doses (Fig. 1d and Fig. 2b). At a dose of 10 kGy, the protein possibly unfolds into non-native states that are prone to aggregation. Intense fluorescence due to bis-ANS bound to the WGA was observed at 10 and 25 kGy while 1 kGy shows significantly less binding (Fig. 2a).

Oligomerisation yields the basis for the multivalency necessary for typical lectin activities (Sharon & Lis, 1993). Therefore, any perturbation in protein structure that may affect the intrinsic activity after irradiation must be clarified. The positive band centred at ~225 nm in the far-UV CD spectra of WGA is characteristic of cystine residues immersed in an asymmetric environment (Drenth, Low, Richardson, & Wright, 1980). Its relatively elevated intensity is due to the high density of disulphide bridges, as well as the lack of secondary-structure repetitive elements. Radiation damage to sulphur-containing amino acids has been reported (Xu & Chance, 2005). This particular effect on disulphide bridges was observed in WGA and suggests that irradiation does not only compromise the dimeric structure but also produces a mixture of partially unfolded species at various stages of unfolding and large amorphous aggregates, after low and high doses of radiation, respectively. Such events were proven by the decrease of intrinsic fluorescence and high binding of bis-ANS to amorphous aggregates.

The current understanding about allergenicity of a plant food protein is determined by a sum of factors, including its abundance,

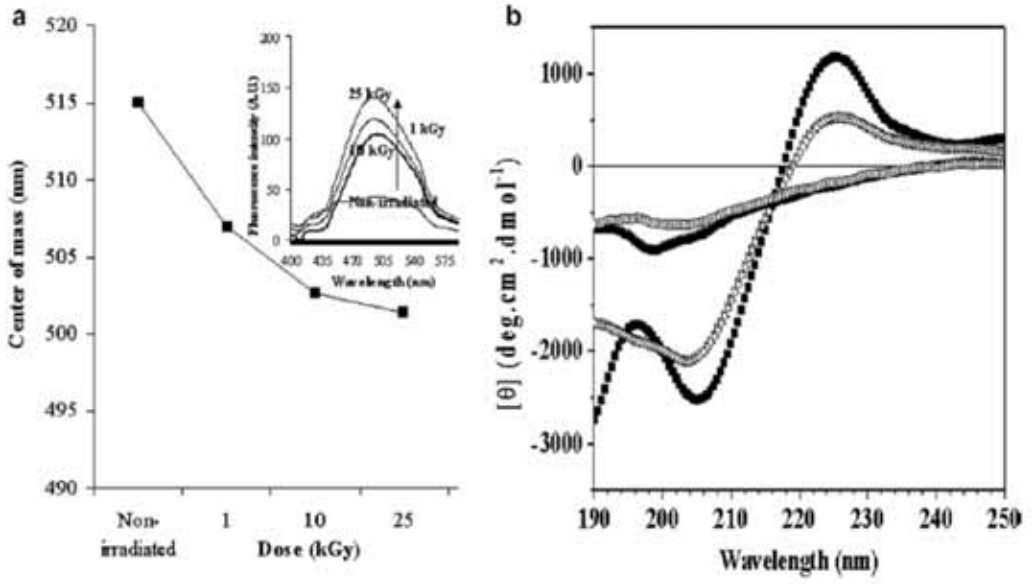

Fig. 2. Ilis-ANS fluorescence and far-UV CD spectra of WGA. (a) Centre of spectral mass of bis-ANS fluorescence; excitation at 360 nm and emission at 400–600 nm. (b) CD spectra were measured in the far-UV range (190–250 nm) in 1-mm path length quartz cuvette. (#) is given in degree squared centimetres per decimole. (■) Non-irradiated and irradiated WGA at (△) 1; (●) 10 and (○) 25 kGy.

Table 1
Body weight, leucocytes and cytokine profiles of mice challenged with native and irradiated WGA.

	Groups			
	G_0	G_1	G_2	G_3
Weight (g)				
Initial	21.6 ± 2.3	27.9 ± 1.0*	22.3 ± 1.3	37.7 ± 2.7*
Post immunisation	40.8 ± 1.1	35.9 ± 0.7*	35.0 ± 1.9*	33.3 ± 5.9*
Post treatment	42.9 ± 1.2	36.7 ± 1.3*	31.8 ± 1.3*,#	32.6 ± 5.1*,#
White blood count				
WBC[a]	1.7 ± 0.4	3.5 ± 0.3	8.6 ± 5.4*,#	5.8 ± 3.1
Large lymphocytes[b]	82 ± 6.6	88.2 ± 6.3*	90.6 ± 5.1*	92.2 ± 2.8*
Monocytes[b]	2.8 ± 2.0	1.6 ± 0.8	1.2 ± 0.8	1.3 ± 0.8
Neurophils[b]	12 ± 3.5	7.3 ± 4.0*	7.1 ± 4.7*	5.3 ± 1.9*
Eosinophila[b]	2 ± 1.2	0.4 ± 0.2	0.12 ± 0.1	0.8 ± 0.1
Cytokines and chemokines				
Eotaxin (pg/mL)	455.6 ± 70.3	1077 ± 74.5*	694.4 ± 1820.7	889.6 ± 298.8
IL-4 (pg/mL)	<0.31	23.2 ± 16.2*	12.4 ± 10.5	8.6 ± 4.4
IL-5 (pg/mL)	17.9 ± 4.2	191.6 ± 87.4*	23.1 ± 12.8#	45.4 ± 28.9
Rantes (pg/mL)	37.8 ± 8.7	68.1 ± 19.6	97.4 ± 46.7	69.9 ± 25.6

G_0, immunised control animals treated with saline; G_1, immunised mice were treated with native WGA; G_2,G_3, immunised mice treated with irradiated WGA at 1 and 10 kGy, respectively.

[a] White blood count in thousand per cubic millimetre.

[b] Mean per cent of total leucocytes.

* $p < 0.05$ compared to control.

\# $p < 0.05$ compared to native WGA.

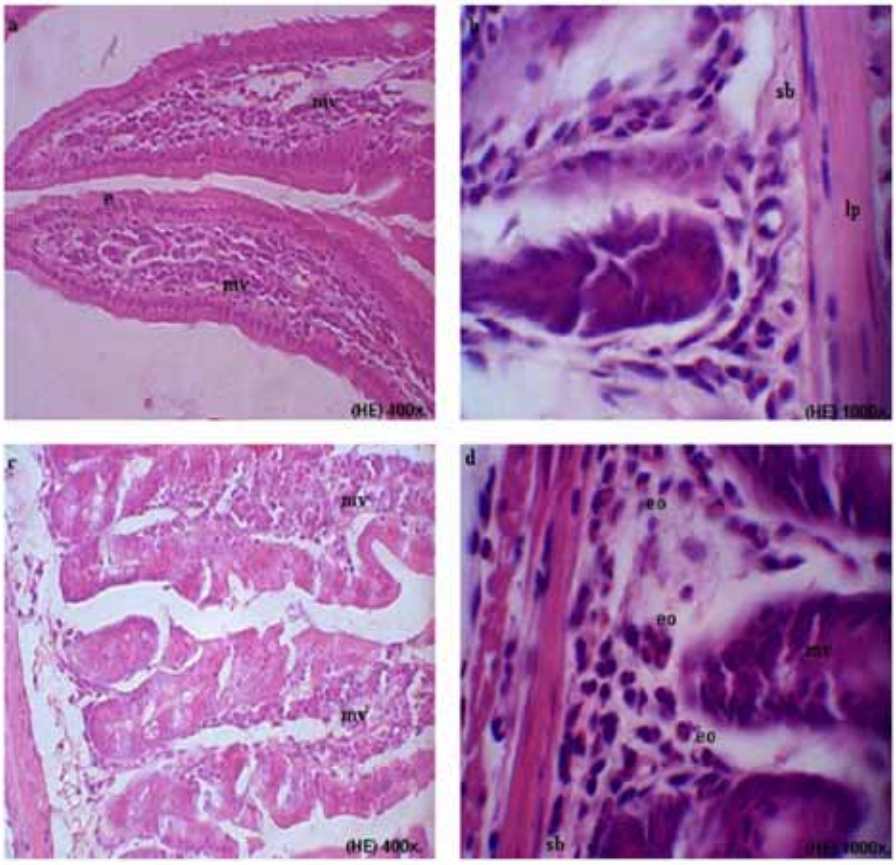

Fig. 3. Photomicrograph of jejunal mucosa of untreated and treated animals with native WGA. (a and b) Jejunum of immunised animals treated with saline: Note the microvilli (mv) coated high enterocytes (e) with a prominent striated cuticle. Central stroma with evident lymphocytic infiltration, submucosa (sb) well defined and preserved lamina propria (lp). (c) Jejunum of animal treated with native WGA: Note the moderate lymphocytic infiltrate filling the stroma of microvilli (mv). (d) Leucocyte infiltration in jejunal submucosa with numerous eosinophils (eo).

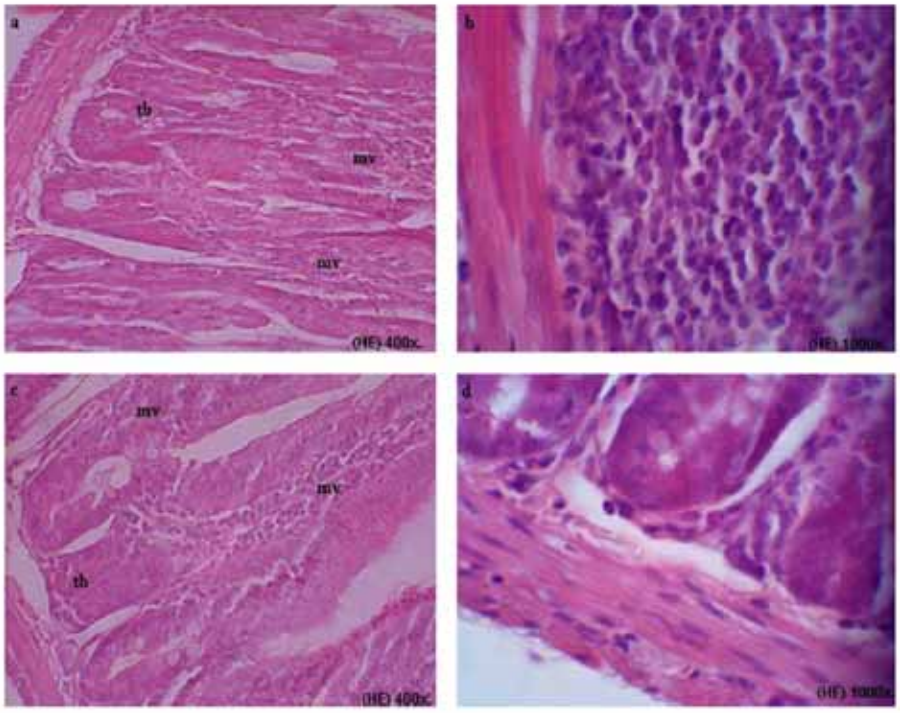

Fig. 4. Photomicrograph of jejunal mucosa of animals treated with irradiated WGA. (a) Jejunum of animals treated with irradiated WGA (1 kGy): Note the dense lymphocyte infiltration in the stroma of the microvilli (mv) and also around the tubular glands or crypts (tb) with numerous polymorphonuclear leucocyte (b). (c and d) Jejunum of animals treated with irradiated WGA (10 kGy) where the following can be observed: the same infiltration already mentioned for 1 kGy with a reduction of lymphocyte infiltration in microvilli (mv) and around the tubular glands (tb) when compared to previous treatments.

the stability to processing and digestion and the protective effect of food matrix (Breiteneder & Mills, 2005b). One aspect of food allergens that remains to be elucidated is the influence of the food matrix on the immune responses to food proteins. It has been hypothesised that the food body, consisting of fats, carbohydrates, and other proteins, may affect allergenic potential of proteins (Van Wijk et al., 2005). The main problem behind the conformational changes of proteins is that these are not always perceived by the general methods of analysis, which complicates the structural analysis if the food matrix is involved. However, these considerations should be investigated to clarify the contribution of food matrix to immune responses against irradiated food allergens.

In addition, since foods are often subjected to a variety of processing conditions, alteration in immunodominant epitopes may potentially affect protein allergenic properties. Processing may destroy existing epitopes on a protein or may generate new ones (neoallergen formation) as a result of change in protein conformation. More often, protein denaturation and/or modification to inactivate epitopes may be a more practical choice to reduce or eliminate food allergens after food processing (Sathe et al., 2005). Through gamma irradiation, we observed relevant functional and structural changes in a dose range above 10 kGy. Thus, we decided to investigate the anti-nutritive effects of irradiated WGA compared to non-irradiated samples. To study in depth the effect of irradiation on food allergenicity, we analysed weight loss, plasma levels of cytokines and leucocytes as well as the histological profile of the gut of animals sensitised and subjected to oral challenge with WGA for 7 days. All results are summarised in Table 1.

A significant ($p < 0.05$) weight loss of animals sensitised and given irradiated WGA was observed when compared with the control group challenged with native WGA. Although different, we note a greater weight loss for animals treated with WGA irradiated at 1 kGy. When blood leucocytes were determined (Table 1), we found increases of leucocytes and lymphocytes in mice treated with irradiated WGA at 1 kGy, being significantly different ($p < 0.05$) from the native-treated and untreated groups. The profile of cytokines revealed an allergic inflammatory response. Animals challenged with native WGA showed a significant ($p < 0.05$) increase of eotaxin, IL-4 and IL-5, when compared to the control group. The animals treated with irradiated WGA, which had weight loss and elevation of leucocytes, showed a significant decrease of IL-5, compared to mice treated with native WGA.

The histological profile of the gut of mice fed on diets containing native WGA was appreciably altered after feeding for 5 days. The jejunal mucosa showed moderate lymphocytic infiltrate filling the stroma of microvilli and a submucosa with numerous eosinophils (Fig. 3c and d) compared to non-immunised animals treated with saline (Fig. 3a and b). Jejunal mucosa of animals treated with irradiated WGA (1 kGy) showed dense lymphocyte infiltration in the stroma of the microvilli and also around the tubular glands or crypts with numerous polymorphonuclear leucocytes (Fig. 4a and b). In irradiated WGA (10 kGy), there was reduced lymphocyte infiltration when compared to previous treatments (Fig. 4c and d). Although we did not observe an association between WGA intake and body weight loss in sensitised animals treated with native WGA, certainly due to the short time of treatment, we can see that the body weight loss and lymphocytic infiltrate in the jejunal mucosa were reduced in the group treated with WGA irradiated at a high dose. Thus, endocytosis or binding to sub-epithelial tissues cannot be observed with irradiated WGA because the agglutination of cells has been abolished.

The increase of cellularity is common in a lectin-rich diet and has been observed (Pusztai et al., 1993). In accord with this, the number of polymorphonuclear leucocytes was higher with the

WGA-containing diet than with the control diet, possibly due to high secretion of eotaxin, IL-4 and IL-5 (Table 1). Eotaxin recruits Th2 cells that in turn produce IL-4 and IL-5, which help to amplify all their effects, resulting in the production of more eotaxin. Significant levels of eotaxin may result in eosinophil recruitment and degranulation, further Th2 recruitment, basophil degranulation and mast cell migration and differentiation (Gutierrez-Ramos, Lloyd, & Gonzalo, 1999). IL-5, acting as a chemokinetic factor for eosinophils, synergises with eotaxin in promoting the fast mobilisation of the eosinophil pool from the bone marrow (Bonecchi et al., 1998). Meanwhile, IL-4 stimulates the differentiation of CD4 + T-cells into Th2 cells, whereas its overproduction is associated with allergies (Tepper et al., 1990). Cell-mediated reactions are also involved, causing mucosal damage, such as crypt hyperplasia and villus atrophy in the late phase (Eigenmann, 2002; Ferguson, 1992). However, these events were not observed after 7 days of continuous challenge.

Although there was a considerable decrease in the allergic inflammatory response of animals challenged with irradiated WGA when compared with native WGA, the results of weight loss were not attuned to those obtained with cytokines in combating to anti-nutritive effects of WGA. However, the loss of intrinsic activity and insoluble amorphous aggregates with lack of native conformational structures was revealed after irradiation. This important finding may irreversibly impair linear and conformational epitopes, not only in the WGA, but also in other classes of food allergens, as observed in milk β-lactoglobulin, chicken egg albumin, and shrimp tropomyosin (Byun, Lee, Yook, Jo, & Kim, 2002). Studies such as ours, which are directed at understanding the mechanisms of food processing on food allergens, are scarce. Therefore, we investigated if food irradiation is safer and more effective in combatting clinical and immunological effects of food allergens. As our food supply becomes increasingly processed and complex, the stability of a protein to food processing may also be important in assessing its allergenic potential.

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6. Considerações finais

Este trabalho investigou a ação da irradiação gama sobre a estrutura molecular e a antigenicidade de alérgenos alimentares, na tentativa de impactar a estabilidade estrutural proteica e de comprometer a integridade de epítopos lineares e conformacionais. Diversas pesquisas têm buscado, por meio de métodos de processamento alimentar, a redução ou eliminação da alergenicidade de alimentos e muito se tem especulado a esse respeito. Os resultados aqui apresentados trazem novas descobertas para a área. Para tal, empregamos um grupo de proteínas vegetais bastante estudadas, as lectinas, as quais exibem características muito similares aos principais alérgenos, como estrutura tridimensional condensada e riqueza de pontes dissulfeto, o que proporciona estabilidade ao calor, aos extremos de pH e a proteases. Descrevemos, pela primeira vez, a desnaturação e fragmentação de alérgenos alimentares pela irradiação em alta dose sem o concomitante incremento de qualquer outro método de processamento.

Para entender o mecanismo de ação desse método físico de tratamento sobre proteínas – neste caso, as lectinas: Cramoll, Con-A e WGA ─, primeiramente, avaliamos a atividade intrínseca em três categorias de dose utilizadas em alimentos, empregando, para isso, a atividade hemaglutinante. Verificamos a completa abolição da atividade em alérgenos submetidos à alta dose de radiação por conta, principalmente, da intensa agregação e precipitação proteica. Mostramos também que os agregados formados apresentam suscetibilidade a agentes caotrópicos (ureia) por meio da dissolução dos agregados e formação de fragmentos peptídicos. Verificamos que a conversão das formas nativas em agregadas envolve uma mudança substancial na estrutura terciária e na superfície hidrofóbica proteica, como foi confirmado por espectroscopia de fluorescência, Dicroísmo Circular (CD) e Calorimetria Diferencial de Varredura (DSC). Além dessas observações, vimos que o Far-UV CD e a fluorescência fornecem evidências importantes para a inexistência de estados de glóbulos fundidos (molten globule) em alérgenos irradiados, uma vez que, em doses elevadas de radiação, a estrutura terciária como também a estrutura secundária não é mantida, o que indica a existência de precipitação em forma de agregados amorfos insolúveis com a falta de estruturas secundárias nativas.

Uma vez compreendido o processo de transição molecular e o tipo de agregado formado, exploramos a modulação da resposta inflamatória alérgica de camundongos sensibilizados e submetidos a um desafio oral com alérgenos irradiados e rastreamos a perda de peso, o perfil de leucócitos, os níveis plasmáticos das citocinas IL-4, IL-5, eotaxina, RANTES e alterações histológicas intestinais. Embora o procedimento de sensibilização não tenha induzido a perda de peso e alterações na contagem de leucócitos plásmaticos, a ingestão contínua de alérgenos não-irradiados reduziu o peso dos animais. Esses experimentos demonstram claramente uma associação entre a ingestão de alérgenos nativos, perda de peso corporal e eosinofilia. Tais efeitos alérgicos foram confirmados pela elevada secreção de eotaxina e exacerbado infiltrado linfocitário e eosinófilico no estroma das microvilosidades e na zona de cólon do jejuno proximal de camundogos sensibilizados. Contrariamente, alérgenos submetidos à alta dose de radiação e que precipitam em forma de agregados amorfos insolúveis revelaram valores significativamente mais baixos de eotaxina e uma proporção de infiltrados linfocitários e eosinofilicos no estroma das microvilosidades muito menor, quando comparado a alérgenos nativos.

Por fim, surpreendentemente, o comportamento alérgico de camundongos desafiados com alérgenos irradiados a baixa dose mostrou uma resposta rigorosamente elevada quando comparada com animais desafiados com alérgenos não irradiados (nativo). Esse fato nos alerta para a possível contribuição do processamento alimentar na exacerbação da notificação de caso de alergia alimentar nas últimas duas décadas. Ficou evidente também a regularidade e abrangência dos resultados, mesmo usando diferentes alérgenos, modelos de experimentação animal e regime de tratamento (agudo e crônico).

Neste esforço aqui apresentado para compreender a ação da radiação ionizante sobre a redução ou eliminação da antigenicidade, ficou a necessidade de melhor caracterizar o impacto sobre epítopos e esclarecer o envolvimento de alérgenos irradiados na modulação da resposta imune intestinal ao concentrar a atenção sobre os potenciais benefícios da irradiação de alimentos como um tratamento alternativo para atenuar a alergenicidade dos alimentos.

7. Conclusão

- Descrevemos, pela primeira vez, a desnaturação e fragmentação de alérgenos alimentares pela irradiação em alta dose sem o concomitante incremento de qualquer outro método de processamento;
- Verificamos a completa abolição da atividade em alérgenos submetidos à alta dose de radiação por conta, principalmente, da intensa agregação e precipitação proteica. Mostramos também que os agregados formados apresentam suscetibilidade a agentes caotrópicos (ureia) por meio da dissolução dos agregados e formação de fragmentos peptídicos;
- Além dessas observações, observamos que o CD e a fluorescência fornecem evidências importantes para a inexistência de estados de glóbulos fundidos (molten globule) em alérgenos irradiados, uma vez que, em doses elevadas de radiação, a estrutura terciária como também a estrutura secundária não é mantida, o que indica a existência de precipitação em forma de agregados amorfos insolúveis;
- Os experimentos demonstraram claramente uma associação entre a ingestão de alérgenos nativos, perda de peso corporal e eosinofilia. Tais efeitos alérgicos foram confirmados pela elevada secreção de Eotaxina e exacerbado infiltrado linfocitário e eosinófilico no estroma das microvilosidades e na zona de cólon do jejuno proximal de camundongos sensibilizados;
- Contrariamente, alérgenos submetidos à alta dose de radiação e que precipitam em forma de agregados amorfos insolúveis revelaram valores significativamente mais baixos de eotaxina e uma proporção de infiltrados linfocitários e eosinofilicos no estroma das microvilosidades muito menor, quando comparado a alérgenos nativos;
- Por fim, surpreendentemente, o comportamento alérgico de camundongos desafiados com alérgenos irradiados a baixa dose mostrou uma resposta rigorosamente elevada quando comparada com animais desafiados com alérgenos não irradiados (nativo). Ficou evidente também a regularidade e abrangência dos resultados, mesmo usando diferentes alérgenos, modelos de experimentação animal e regime de tratamento (agudo e crônico).

Apêndice I

Artigos complementares publicados durante o doutorado

ARTICLE IN PRESS

Radiation Physics and Chemistry ▮ (▮▮▮▮) ▮▮▮–▮▮▮

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Inactivation and fragmentation of lectin from *Bothrops leucurus* snake venom by gamma irradiation

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ABSTRACT

Gamma radiation alters the molecular structure of biomolecules and is able to mitigate the action of snake venoms and their isolated toxins. The effect of γ-radiation on the folding of *Bothrops lecurus* venom lectin was measured by a hemagglutinating assay, intrinsic and bis-ANS fluorescence. Intrinsic and bis-ANS fluorescence analyses indicated that irradiation caused unfolding followed by aggregation of the lectin. Our results suggest that irradiation can lead to significant changes in the protein structure, which may promote the loss of its binding property and toxic action.

1. Introduction

Snake venoms are complex mixtures of bioactive proteins and polypeptides (Koh et al., 2006). These toxins are enzymatic and non-enzymatic proteins and synergistic interactions between venom proteins enhanced the lethal potency of the snake venom. Complexes of protein families, such as metalloproteases, serine proteases, C-type lectins (CTLs), C-type lectin-related proteins (CLRPs) and three-finger toxins (3FTxs), have also been reported in venoms (Doley and Kini, 2009). Lectins are proteins or glycoproteins that are ubiquitous in nature and bind reversibly to carbohydrates (Sharon and Lis, 2004).

Ionizing radiation causes changes in the function and integrity of biomolecules, including proteins, by two different means: first, by interacting directly on proteins (Kempner, 2001) and second, by the formation of major products from radiolysis of water (Wang and Wang, 2007). However, the effect induced after water radiolysis represents 90% of the molecular radiation-induced damage. The exposure of proteins to low doses of radiation produces chemical and physical damage that may result in changes to the protein's primary, secondary or tertiary structure, yet keeping their immunological properties intact (Nascimento et al., 1996).

The intimate relationship existing between the structure and activity of proteins has received attention from researchers. Many studies have shown the effects of gamma radiation at the molecular level, including on the biological activity of snake venom, where radiation results in a decrease or loss of enzymatic and toxic actions (Casare et al., 2006). BlL is a galactoside-binding lectin from *Bothrops leucurus* snake venom. The lectin, a protein of 30 kDa composed of two subunits of 15 kDa, has antibacterial activity against Gram-positive bacteria (Nunes et al., 2011). Here, the interest lies in studying the effects of ionizing radiation on a lectin isolated from the venom of *Bothrops leucurus*, in order to employ these results in the explanation of how radiation affects the structure-activity relationship of snake venom lectin.

2. Experimental

2.1. Purification of *Bothrops leucurus* lectin (BlL) and lectin irradiation

Bothrops leucurus lectin (BlL) was purified according to Nunes et al. (2011). The lectin aliquots (0.07 mg/mL), in phosphate buffer (pH 7.0) and borosilicate glass vials (16–125 mm), were frozen and irradiated under atmospheric O_2 using a Gammacell

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Inflammopharmacology

RESEARCH ARTICLE

A novel antimicrobial lectin from *Eugenia malaccensis* that stimulates cutaneous healing in mice model

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Abstract
Objective The present work reports the purification and partial characterization of an antibacterial lectin (EmaL) obtained from *Eugenia malaccensis* seeds as well as the evaluation of its effect in the daily topical treatment of repairing process of cutaneous wounds in mice.
Materials and methods The cutaneous wound was produced by the incision of the skin and use of lectin in the treatment of mice cutaneous wounds was evaluated. Surgical wounds were treated daily with a topical administration of EmaL and parameters such as edema, hyperemia, scab, granulation and scar tissues as well as contraction of wounds were analyzed.
Results A novel lectin, with a molecular mass of 14 kDa, was isolated from *E. malaccensis* using affinity chromatography. The lectin (EmaL) agglutinated glutaraldehyde-treated rabbit and human erythrocytes; the lectin-induced rabbit erythrocyte agglutination was inhibited by glucose, casein, ovalbumin and fetuin. Also, Emal was very effective in the inhibition of bacterial growth, with the best inhibition results obtained for *Staphylococcus aureus*. Inflammatory signals such as edema and hyperemia were statistically less intense when EmaL was applied compared to the control. The histopathological analysis showed that the treated injured tissue presented reepithelialization (complete or partial) and areas of transition more evidenced than those of the control group, especially due to well organized pattern of collagen fibers presented in the granulation fibrous tissue.
Conclusion Presented results are a preliminary indication of the pharmacological interest in using EmaL as antimicrobial agent and in the repairing process of cutaneous wounds.

Keywords *Eugenia malaccensis* · Lectin · Antibacterial · Cutaneous wound healing

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Introduction

Lectins constitute a heterogeneous group of non-immune protein, structurally distinct with two or more specific binding sites to mono or oligosaccharides (Barondes 1988). They are purified from different species (Sharon and Lis 2004) and in plants they are mainly obtained from legume seeds (Konozy et al. 2003). They have attracted great interest because of their various biological activities (Peumans and Van Damme 1998), in particular, the interactions of plant lectins with human pathogenic bacteria (Slifkin and Doyle 1990). Lectins demonstrated the binding capacity to a wide variety of complex carbohydrates such as teichoic acid, teichuronic acids, peptidoglycans and lipopolysaccharides present in cell walls (Pistole 1981;

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Lectin from *Crataeva tapia* bark exerts antitumor, anti-inflammtory and analgesic activities

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Abstract: *Crataeva tapia* bark lectin was evaluated for its antitumor activity against sarcoma 180 in Swiss albino mice. The anti-inflammatory and analgesic properties were investigated in models of inflammation and nociception. The anti-inflammatory assay was induced by carrageenan induced peritonitis and the analgesic activity was induced by acetic acid-induced writhing response. The lectin presents low toxicity with a LD_{50} of 2,500 mg/kg body weight and significant antitumor activity causing inhibition of tumor growth. The lectin also promoted significant reduction (35.4%) in the number of neutrophil migration induced by carrageenan. Concerning its analgesic property, the lectin inhibits abdominal contractions induced by acetic acid. The current results revealed a lectin with significant antitumoral, anti-inflammatory and antinociceptive activities. Further investigations to unveil the exact mechanisms are needed.

Keywords: *Crataeva tapia*, lectin, antitumoral, anti-inflammatory, antinociceptive

Introduction

Plants have been a source of medicines for humans since pre-history and it is notable that in the last four decades, there has been a resurgence of interest in the study and use of medicinal plants. Natural products derived from plants have been characterized and identified as new chemical compounds of therapeutic importance. Lectins are proteins that can recognize and reversibly bind to carbohydrates or other substances derived from sugars[1]. Nowadays, it is well known that despite their small size, sugars play roles in storage or in relaying information within or between cells. Due to their carbohydrate-specific interaction, lectins have been versatile and useful molecular tools for the study of glycoconjugates on the cell surface. Therefore are excellent candidates to be explored in cancer research and as therapeutics agents[3–5].

Inflammation is part of the complex biological response of vascular tissues to harmful stimuli, such as pathogens, damaged cells, or cancer. Inflammation is usually associated with pain as a secondary process resulting from the release of analgesic mediators[6]. Non-steroidal anti-inflammatory agents – NSAIDS have been prepared and marketed[6], however, these drugs are known to provoke gastrointestinal irritation[7]. Thus, recognition of inflammatory processes incidence caused by cancer robustly correlates with an increasing attention on safety and effectiveness of novel pharmaceuticals. The aim of the present study was to investigate the antitumoral, anti-inflammatory and antinociceptive properties of bark lectin from *Crataeva tapia* on experimentally induced tumors, inflammation and pain.

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Material and methods

Purification of Crataeva Tapia Bark Lectin. *Crataeva tapia* bark was obtained through a sequential purification protocol according to Nascimento et al.[8]. Bark powder (10g) was mixed with150 mM NaCl (100 ml). An extract was obtained by agitation of the mixture overnight at 4°C. The extract was filtered through gauze and underwent centrifugation at 4,000 g for 15 min. The supernatant was termed crude extract (CE). The reversed micellar system was constituted using the anionic surfactant, sodium di(2-ethylhexyl)sulfosuccinate in isooctane. Extraction and back-extraction procedures were performed as follows: (1) to buffered lectin preparations (3 ml) containing

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Scientific Research

Stability of the Antioxidant Activity of Flavonoids after Gamma Irradiation

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ABSTRACT

Food irradiation has the purpose of destroying insects or microorganisms, thereby increasing the safety and shelf life of foods. Flavonoids are ubiquitous plant secondary products with radical scavenger ability. In the present study their antioxidant stability after gamma irradiation was evaluated. The flavonoids showed fast scavenger ability measured with the 2.2-diphenyl-1-picrylhydrazyl radical (DPPH) after high doses of radiation. The low damage caused by irradiation on antioxidant capacity of the flavonoids shows their potential use in combating chemical oxidation of biomolecules in irradiated foods.

Keywords: *Flavonoid, Food Irradiation, Scavenger Capacity, Gamma Irradiation*

1. Introduction

Radiation treatments of biological materials have been applied for various processes: sterilization of materials, reduction of microbial contamination of food, and increasing the safety and shelf life of foods. Gamma irradiation can damage biomolecules directly by rupturing covalent bonds as a result of transfer of photon energy, and indirectly, by producing free radicals and other non-radical reactive oxygen species [1]. Investigations have shown that free radicals cause chemical oxidation [2] and oxidative stress [3] which makes the recovery of biologically active materials unlikely.

Flavonoids are among the most ubiquitous of plant secondary metabolites and aid in the reproduction and protecttion of plants [4]. Their low toxicity and high antioxidant activity [5] have been documented. The evaluation of the radioprotective effects of flavonoids on organoleptic properties, sensory and cytological has been described in strawberries [6]. Recently, some studies showed the radioprotective effects of flavonoids in gamma-irradiated mice [7-9]. Irradiated flavonoid solutions produce chemical reactions between the solute and reactive species, which are the result of the radiolyzed solvent operating to quickly repair the chemical modifications to biomolecules [10,11]. One of the most important probes for evaluating the fast repair of antioxidant capacity is the 2.2-diphenyl-1-picrylhydrazyl radical (DPPH), a stable nitrogen synthetic radical [12]. Therefore, the aim of our study was to evaluate the free radical scavenging ability and radiostability of flavonoids isolated from *S. jacobinensis* bark after a high dose of γ-radiation.

2. Materials and Methods

2.1. Flavonoid Isolation

S. jacobinensis bark was collected from trees in the semi-arid region of the state of Pernambuco, Brazil. *S. jacobinensis* dry bark was mixed in a beaker with 250 ml of ethanol/acetic acid/water (70:4:26, v:v:v) for 2 h at 4°C. The extract containing flavonoids was chromatographed in an anion-exchange DEAE-Cellulose (Sigma) column (40 × 2.5 cm) equilibrated with ethanol-water (70:30, v:v). Elution was performed with ethanol-acetic acid-water (70:4:26, v:v:v) at a flow rate of 2 ml/min. Each fraction collected was checked by thin layer chromatography (TLC) to determine their compositions using ethyl acetate/formic acid/acetic acid/water (100:11:11:26, v:v:v:v). Vanillin dipping in hydrochloric acid was used for detection under UV (254 nm). TLC conditions were

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Letter to the Editors-in-Chief

A new exogen anticoagulant with high selectivity to intrinsic pathway of coagulation

Dear Editors,

Venous thromboembolism (VTE) is an important clinical problem with an incidence of 1–2 per 1000 persons and the consequences can be life-threatening [1]. Although the causes are not always identified, it is believed that increasing hypercoagulability can alter the balance of hemostasis, explaining the occurrence of VTE in apparently healthy individuals [2]. Hypercoagulability may be due to defective naturally occurring anticoagulant mechanisms or to heightened levels of procoagulant factor [3]. These factors belong to the clinical intrinsic pathway of blood coagulation, which is cumulatively monitored by the coagulation and activated partial thromboplastin time (APTT) tests [4].

Traditionally, anticoagulant therapy is used for prevention and treatment of VTE. As endogenous or exogenous anticoagulants interfer with the coagulation factors, blood coagulation can be prolonged or stopped [5]. Such anticoagulants have been convenient tools for exploring the complex mechanisms of the coagulation cascade, and coincidentally, as an object of research for possible therapeutic purposes [6]. For both, additional validation of developed products and extensive clinical trials are performed globally to validate claims of the safety and efficacy of newer drugs. In the search for new anticoagulant agents for anticoagulant therapy, a number of C-type lectin-like proteins have been described. Anticoagulant lectins have also been isolated from snake venoms with action on thrombosis and hemostasis by inhibiting or activating specific platelet membrane receptors and blood coagulation factors [7]. In this study, we describe the action of *Crataeva tapia* bark lectin on blood clotting time which may act as an anticoagulant alternative to conventional drugs in polytherapeutic approaches.

Crataeva tapia bark was obtained through a sequential purification protocol according to Nascimento et al, (2008) [8] and stored at −20 °C. Activated partial thromboplastin time (APTT) and prothrombin time (PT) were determined in an automated coagulometer ST4 (Diagnostica Stago, France). The test plasma utilized was obtained commercially (Instrumentation Laboratory, Milano, Italy). Test plasma and calcium chloride were pre-heated to 37 °C. For the PT, the negative control was made with 50 µl of test plasma, incubation for 60 s, with subsequent addition of 100 µl of reagent (recombinant thromboplastin) from Instrumentation Laboratory (Milano, Italy). CrataBL at different concentrations (1.25; 2.5; 5.0; 10.0; 20.0 and 40 µg) was incubated (60 s) in 50 µl of test plasma, with subsequent addition of 100 µl of reagent. For the APTT, the control tube containing 50 µl of test plasma and 50 µl of APTT reagent from Instrumentation Laboratory (Milano, Italy) or test tubes containing CrataBL (same concentrations used to PT), 50 µl of test plasma and 50 µl of APTT reagent were incubated for 120 s at 37 °C. Subsequently, 50 µl of 0.025 M calcium chloride was added to the tubes. For thrombin time (TT) measurement, 50 µl of test plasma was incubated for 120 s in 100 µl of bovine thrombin (15 UNIH – International Unit of Hematology) from Instrumentation Laboratory (Milano, Italy). CrataBL at the same concentrations mentioned above were incubated (120 s) in 50 µl of test plasma, with subsequent addition of 100 µl of thrombin (15 UNIH). The assays were made in triplicate and the results were expressed as the average of measurements for each sample.

The APTT was determined for factors VIII (Diagnostica Stago, France), IX (Dade Behring, USA), XI or XII (Diamed, Switzerland), and deficient plasma (FVIII, FIX, FXI and FXII). The control was made with deficient plasmas (FVIII, FIX, FXI and FXII), 50 µl normal plasma and 50 µl APTT (Instrumentation Laboratory, Italy), incubation for 180 s at 37 °C with subsequent addition of 0.025 M calcium chloride (50 µl). CrataBL (50 µl) at the above mentioned concentrations were pre-incubated with 50 µl of FVIII, FIX, FXI or FXII deficient plasma, 50 µl of normal plasma and 50 µl of APTT reagent for 180 s at 37 °C. The 0.025 M calcium chloride solution (50 µl) was then added and the clotting time recorded. The experiments were performed in triplicate.

The inhibitory activities on bovine trypsin and human plasma kallikrein (huPK) proteinases were determined by measuring the remaining hydrolytic activity towards synthetic specific substrates Bz-Arg-pNan and H-D-Pro-Phe-Arg-pNan, respectively. The pre-incubation of the enzyme / CrataBL was performed in 0.05 M Tris–HCl buffer, pH 8.0, 37 °C containing 0.02% CaCl2, and 0.1 M Tris–HCl buffer containing 0.5 M NaCl, pH 8.0, 37 °C for trypsin and kallikrein, respectively. After pre-incubation for 10 min, enzyme or enzyme-lectin were added to the substrate solutions for a final volume of 200 µL. The K_{iapp} values were determined by adjusting the experimental points to the equation for tight binding, using a nonlinear regression, with the Grafit 3.01 program [9]. The results were expressed as mean ± S.E. Data for the various parameters were compared and statistically assessed using one-way ANOVA (Origin® 6.0), followed by the post hoc Bonferroni test, with a significance level of $p<0.05$.

A variety of new anticoagulants are being developed and tested to inhibit the various steps in the coagulation cascade [10]. Traditionally, two main pathways have been described for blood coagulation: the intrinsic or contact pathway and the extrinsic or tissue factor pathway [11]. In order to clarify the effects of CrataBL on 'intrinsic' and 'extrinsic' coagulation, we performed PT and APTT assays. Although change with a statistical significance has been observed, the PT remained within normal limits at all concentrations of CrataBL. In contrast, the APTT was significantly prolonged (to more than 300 s) with a dose-dependent response (Table 1). These results shows that the lectin inhibited the intrinsic pathway and had insignificant effects on the extrinsic pathway which suggested that CrataBL had a dominant effect on one or more of the intrinsic factors rather than those of the extrinsic pathway. As reported in previous studies [12], a C-type lectin-like protein from snake (*Agkistrodon acutus*) venom also prolonged APTT to prolonged more than 300 s, similar to that obtained in our study.

CrataBL did not prolong the normal clotting time by the TT measurement (Table 1). This is a direct measure of fibrinogen function which is prolonged in hypofibrinogenemic states or if an abnormal fibrinogen (dysfibrinogenemia) is present.

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Short communication

Occurrence and effect of propanedial on top-fermenting yeast

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ABSTRACT

Peroxidation of polyunsaturated fatty acids caused by free radicals compromise the integrity of biological membranes. Propanedial is identified as the main product formed via the decomposition of lipid peroxidation products. Propanedial has been inferred to have mutagenic and cytotoxic roles. Top-fermenting yeast is responsible for converting fermentable sugars into alcohol. In the present paper we evaluate the relationship of lipid peroxide levels and the degree of impairment in glucose consumption of *Sacharomyces cerevisiae* cells. Results showed that cell suspensions pre-incubated with Propanedial reduced glucose consumption by about 30% resulting in a decrease in the yield of top-fermenting yeast. These findings suggest that Propanedial affects the fermentation process of *S. cerevisiae*.

1. Introduction

In the traditional ethanol process, brewer's yeast is propagated under weakly aerated conditions [1]. Oxygen is needed for the synthesis of sterols and unsaturated fatty acids, which are vital elements of cell membranes [2]. Without a supply of these lipids, the cells cannot reproduce and their viability is lessened. Free radicals and reactive oxygen species (ROS) are generated from wort oxygenation and within the cell during the limited period of yeast aerobic metabolism [3]. These components result in cell damage and potentially represent significant stress to yeast [4].

The oxidation of polyunsaturated fatty acids by ROS found in cell membranes plays a role known as lipid peroxidation, which can be measured and used as an indicator of cellular oxidative stress [5]. The products from peroxidation accumulated during aerobic metabolism induce modifications in the structure, fluidity and permeability of the membranes [8]. The oxidation of polyunsaturated fatty acids serves as a convenient index for the extent of peroxidation reactions. Among the thiobarbituric acid reactive substances (TBARS) formed, Propanedial is identified as the main product. The high content of unsaturated lipids, the aerobic conditions and the presence of metal ions are promoters of peroxidation processes [6].

Yeasts are single-celled microorganisms that reproduce by budding. They are responsible for converting fermentable sugars into alcohol and other products. *Saccharomyces*

Abbreviations used: ROS, Reactive oxygen species; TBARS, Thiobarbituric acid reactive substance assay.

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Purification of a lectin with antibacterial activity from *Bothrops leucurus* snake venom

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ABSTRACT

A novel lectin was isolated from *Bothrops leucurus* snake venom using a combination of affinity and gel filtration chromatographies. The lectin (BlL) agglutinated glutaraldehyde-treated rabbit and human erythrocytes with preference for rabbit erythrocytes. Galactose, raffinose, lactose, fetal bovine serum and casein inhibited lectin-induced rabbit erythrocyte agglutination. BlL, with a molecular mass of 30 kDa and composed of two subunits of 15 kDa, showed dependence on calcium. BlL is an acidic protein with highest activity over the pH range of 4.0–7.0 and stable under heating to 70 °C. Fluorescence emission spectra showed tryptophan residues partially buried within the lectin structure. The percentages of secondary structure revealed by circular dichroism were 1% α-helix, 44% β-sheet, 24% β-turn and 31% unordered. BlL showed effective antibacterial activity against Gram-positive bacteria *Staphylococcus aureus*, *Enterococcus faecalis* and *Bacillus subtilis* with minimal inhibitory concentrations of 31.25, 62.25 and 125 μg/mL, respectively. In conclusion, *B. leucurus* snake venom contains a galactoside-binding lectin with antibacterial activity.

1. Introduction

Lectins are proteins or glycoproteins that bind reversibly to carbohydrates and glycoconjugates (De-Simone et al., 2006). Lectins have been found in a wide range of organisms from microorganisms to plants and animals (Utarabhand et al., 2007). C-type lectins are a large family of Ca^{2+} dependent lectins. Animal C-type lectins can be classified into 17 groups according to structural and functional characteristics (Zelesnky and Gready, 2005). Snake venoms contain C-type lectins included in group VII, which are true sugar-binding lectins composed of homodimers or homooligomers and with Ca^{2+} and usually galactose-binding properties (Clemetson, 2010). Snake venoms also contain C-type lectin-like proteins which are heterodimers or oligomeric complexes of heterodimers called snaclecs (snake venom C-type lectins); this group is more abundant and possesses a loop-swapping or higher-order multimerization (Ogawa et al., 2005; Clemetson et al., 2009; Clemetson, 2010).

Snake venom lectins are able to inhibit or activate specific platelet membrane receptors and blood coagulation factors (Morita, 2004, 2005; Ogawa et al., 2005; Wang, 2008) and can promote a diversity of biological effects, such as lymphocyte proliferation (Mastro et al., 1986), induction of edema (Lomonte et al., 1990; Panunto et al., 2006), induction of Ca^{2+} release from the sarcoplasmic reticulum (Ohkura et al., 1996), inhibition of cancer cell proliferation (Pereira-Bittencourt et al., 1999), erythrocyte agglutination *in vitro* (Kassab et al., 2001), cytotoxicity to tumors and endothelial cell lines (Carvalho et al., 2001), renal effects (Havt et al., 2005) and induction of rolling of leukocytes (Elifio-Esposito et al., 2007).

Glycoconjugates present on bacterial cell surfaces, such as peptidoglycans, lipopolysaccharides and teichoic acids, constitute potential lectin targets (Lee et al., 1998; Santi-Gadelha et al., 2006). Recently, it was reported that snake venom lectins are able to inhibit growth of phytopathogenic bacteria (Rádis-Baptista et al., 2006; Barbosa et al., 2010); however, the interactions between snake venom lectins and human pathogenic bacteria have not been studied.

Bothrops leucurus (white-tailed-jararaca) is an important venomous snake that inhabits northeastern Brazil. *B. leucurus* was responsible for all cases of envenomation from snakebite recorded in the metropolitan region of Salvador (State of Bahia, northeastern Brazil) from January to June 1990 (Lira-da-Silva and Nunes, 1993) and an epidemiological study in Bahia in 2001 revealed that this species was responsible for all confirmed cases of envenomation by *Bothrops*

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Rapid Communication

Gamma irradiation as an alternative treatment to abolish allergenicity of lectins in food

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ABSTRACT

Food irradiation is the process of exposing food to ionising radiation to eliminate microorganisms or insects. Lectins are the most common agents in food intolerance, and efficient methods to reduce unwanted or intolerant immunological effects of lectins have not yet been described. *Sebastiania jacobinensis* bark lectin was structurally altered after gamma irradiation. Hemagglutination assays showed that the lectin was stimulated by low doses of radiation (0.1 kGy), while high doses (above 1 kGy) induced a significant loss of activity. The effect of γ-radiation on lectin hydrophobicity was measured by intrinsic and bis-ANS fluorescence. High doses of ionising radiation suppressed the intrinsic fluorescence emission and promoted polypeptide fragmentation and hydrophobic surface modification. The results suggested that changes in the hydrophobic surface induced by gamma irradiation led to protein misfolding and, subsequently, to aggregation. The pioneering viewpoints presented on the stability of a food allergen after gamma irradiation might contribute to the development of harmless and more effective methods to reduce or eliminate food allergenicity.

1. Introduction

Reliable estimates of the prevalence of food allergies/intolerance or the relative importance of the most common food allergens do not exist. Results from epidemiological studies, combined with knowledge on systemic reactions to contact allergens and celiac disease, have suggested that the prevalence of food allergies/intolerance in the adult European population is approximately 5% (Madsen, 1997), demonstrating the great importance of food allergies/intolerance in the health policy literature. Lectins are proteins or glycoproteins that are ubiquitous in nature and have one or more carbohydrate binding sites that lack catalytic function or immunological characteristics (Sharon & Lis, 2004). Most plants contain lectins that can be toxic, inflammatory or both and cause food intolerances or sensitivities. High levels of lectins are found in grains, legumes (beans and peanuts), dairy products and plants in the nightshade family. The total dietary intake of lectins in vegetarian diets rich in leguminous seeds is about 0.1–10 g/kg (Peumans & Van Damme, 1996). While lectins are present in most foods, those that contain higher levels are problematic for many genetically vulnerable individuals. Previously described methods to reduce unwanted or intolerant immunological effects of lectins have been inefficient (Sathe, Teuber, & Roux, 2005) because many lectins are resistant to cooking and digestive enzymes. These aspects are responsible for reports of aggravation of inflammation, digestive diseases and the emergence of food allergies. Lectins bind to specialised intestinal cells (crypt cells) in the mucosa of the duodenum through the carbohydrate recognition domain (CRD), which causes deleterious effects on digestion (Gupta & Sandhu, 1997).

Food irradiation is the process used to eliminate insects, fungi or bacteria that spoil food or cause human disease (Farkas, 1998). Because irradiation kills disease-causing bacteria and reduces the incidence of food borne illnesses, hospitals sometimes use irradiation to sterilise food for immunocompromised patients. Radiation treatments of biological materials have also been applied to various processes, such as the sterilisation of medical supplies. However, high doses of gamma radiation may affect the functional integrity of biomolecules. Protein irradiation promotes extensive structural damage and abolishes biological activity (Davis, Parniak, Kaufman, & Kempner, 1997) through two different mechanisms. First, it splits covalent bonds in target proteins by direct photon energy (Kempner, 2001); second, via water radiolysis, it indirectly produces reactive oxygen species (ROS) responsible for the

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Biocontrol of *Fusarium* species by a novel lectin with low ecotoxicity isolated from *Sebastiania jacobinensis*

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ABSTRACT

A lectin from *Sebastiania jacobinensis* bark was isolated using a combination of acetone precipitation, ammonium sulphate fractionation, ion exchange and gel filtration chromatographies. The lectin purified, with a molecular mass of 52.0 kDa and composed of two subunits of 24 kDa, is a glycoprotein with a neutral carbohydrate content of 6.94%. The lectin shows maximum activity over the pH range 4.0–7.5 and heat stability up to 70 °C. Our results show that the lectin is an incompetitive inhibitor for trypsin, with a *Ki* of 0.39 ± 0.02 μM. Fluorescence spectroscopy indicated the existence of a hydrophobic surface. The percentages of secondary structure are 75% α-helix, 10% β-sheet, 5% β-turn and 10% unordered. Lectin inhibits the mycelial growth of *Fusarium moniliforme* and *Fusarium oxysporum* with an IC_{50} value of 123 ± 0.5 and 303 ± 0.9 μg, respectively. *Artemia salina* Leach and embryos of *Biomphalaria glabrata* are not affected by the lectin, indicating low environmental toxicity. Alternative viewpoints are presented that might hopefully help in future efforts to develop safer and more effective microbial control agents.

1. Introduction

Increasing awareness of the potential impacts of crop-protection agents or pesticide use has led to the development of research with natural products to ensure that risks to man and the environment are limited. Thus, the regulation of natural products as crop-protection agents should have to undergo the same procedure as for a conventional chemical product (Neale, 2000). Antimicrobial agents are provided for control of certain diseases of wheat and other cereals caused by *Fusarium* species, including *Fusarium* head blight of wheat and other cereals. These agents can also improve yield of wheat plants and cereals. Plant lectins are a heterogeneous group of proteins or glycoproteins that share the capacity to identify a specific carbohydrate. Their widespread distributions in the plant kingdom suggest a physiologically important function (Sharon, 2007). They have attracted great interest because of their various biological activities, such as antiproliferative, antitumour, antifungal and antiviral properties (Peumans & Van Damme, 1998). Seeds, especially of leguminous species, are common sources of lectins, but they are also present in latex and bark of different species (Branco et al., 2004; Wititsuwannakul, Rukseree, Kanokwiroon, & Wititsuwannakul, 2008). The proteins provide an opportunity for discovery and a starting point for optimising complex cellular processes and molecular mechanisms. Providing rigorous and comprehensive characterisations for these proteins is invaluable to researchers and frees them to confidently pursue creative experimentation. Circular dichroism (CD) spectroscopy can be a valuable method for determining the secondary structures of proteins (Johnson, 1999). Intrinsic fluorescence and 4,4′-Bis-1-anilinonaphthalene-8-sulphonate (Bis-ANS) have been used in folding, stability studies and as evidence of conformational change in proteins, by assessing hydrophobic regions (Hawe, Sutter, & Jiskoot, 2008).

The salt-water crustacean, *Artemia salina* Leach, is used as food for fish. *A. salina* is sensitive to the effect of active substances and its mortality is used to monitor toxicity because it is highly sensitive to many chemical substances (Almeida, Silva, & Echevarria, 2002). *Biomphalaria glabrata* – Say, 1818 – is a snail of the Planorbidae family with a wide distribution in Brazil. The ease of observing antibiotic effects of drugs on embryonic development of *B. glabrata* and mortality of *A. saline* has urged their use as a way to monitor the environmental impact and selectivity of microbiological control agents (Mclaughlin & Rogers, 1998; Münzinger,

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A new mistletoe *Phthirusa pyrifolia* leaf lectin with antimicrobial properties

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ABSTRACT

Phthirusa pyrifolia leaf lectin (PpyLL) was obtained from the hemi-parasitic medicinal plant mistletoe through saline saturation and two consecutive chromatographic steps on Sephadex G-100 and ion exchange on CM-cellulose. SDS-PAGE of the protein under non-reducing conditions revealed a monomeric protein with a molecular weight of 15.6 kDa. Under reducing conditions in the presence of 2-mercaptoethanol, the protein showed two bands with molecular weights of 15 and 7 kDa. PpyLL, an acidic glycoprotein with 19% sugar content, was not dependent on divalent cations. It was stable up to 70 °C and exhibited maximum hemagglutination at pH 7.5. Lectin fluorescence emission spectra at different temperatures showed that the lectin fluorescence increased when the temperature increased. PpyLL showed a unique affinity for the phosphate derivative of fructose, fructose-1-6-biphosphate. PpyLL showed effective antimicrobial activities against bacteria (Gram-positive: *Staphylococcus epidermidis*, *Streptococcus faecalis* and *Bacillus subtilis*; Gram-negative: *Klebsiella pneumoniae*) and fungi (*Fusarium lateritium* and *Rhizoctonia solani*). Therefore, PpyLL specificity, as determined by a new sugar affinity, may be significant to determine its biological potential.

1. Introduction

Lectins are naturally occurring proteins/glycoproteins with substantial structural diversity; they bind carbohydrate residues selectively and non-covalently and are involved in various biological processes. Lectins recognize sugar determinants in the wall or in the capsule of bacteria and have been suggested to participate in the innate immune response. This may be accomplished by inducing bacterial agglutination or acting as opsonins, which enhances the phagocytosis rates of microorganisms [1]. Some lectins may have affinity for carbohydrates but are not specific; it is not clear how lectins can identify several sugars present in different positions in bacteria, fungi, enzymes and other glycoproteins. The usual methods applied to purify lectins are saturation with ammonium sulfate, affinity chromatography [2], ion exchange chromatography [3] and liquid–liquid extraction using reversed micelles [4]. Recently, lectin biology has applied these tools in the biomedical field and also in the treatment of diseases, including cancer [5]. Another application reported in the literature involving lectins is their antimicrobial activity [6], where lectins may act against microorganisms by interfering with their growth and playing a role in defense systems [7].

Mistletoes comprise about 900 species in 70 genera that are mainly distributed in tropical areas of Africa, Southwest Asia and South America [8]. *Phthirusa pyrifolia*, well known in Brazil as "erva-de-passarinho", belongs to the Loranthaceae family and is a hemi-parasite plant that parasitizes a broad range of gymnosperms and angiosperms. The traditional medicine in developing countries uses a wide variety of natural products in the treatment of common infections. Research on the compounds present in aqueous extracts from mistletoe, including lectins, and their biological properties gained special attention due to their immune modulated capacity for improving natural killer cell activities. Moreover, mistletoe lectins participate in the apoptosis of tumor cells *in vitro* and act as adjuvants in bladder cancer treatment [9]. However, not much is known about mistletoe lectin. The European mistletoe *Viscum album* is a hemi-parasitic plant that is widely spread over Europe and parts of Asia and contains at least three lectin isoforms (ML-I, ML-II and MI-III); it has been suggested that ML-I is part of a defense system against insects, bacteria and fungi [10]. Mistletoes usually have slower rates of photosynthesis than their hosts. Seasonal variations in the availability of water, light and nutrients in tropical forests have the potential to limit the productivity of the plant components [11].

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