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AVALIAÇÃO DE HIDROLISADOS DE CASEÍNA COMO ANTIOXIDANTES EM PRODUTOS CÁRNEOS E CHOCOLATE BRANCO

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RESUMO

Estudos recentes indicam que os peptídeos obtidos pela hidrolise enzimática da caseína podem apresentar atividades antioxidantes. Neste trabalho, previamente obteve-se os peptídeos através de hidrolise da caseína utilizando as enzimas Alcalase e Flavourzyme (4h, a 50ºC e pH 8), selecionando os que apresentaram as melhores características, *in vitro*, relativas à atividade antioxidante. A hidrolise enzimática utilizando a enzima Flavourzyme mostrou melhores resultados, com alto valor de proteína solúvel e conteúdo de aminoácidos livres, além de peptídeos de menor peso molecular do que com a Alcalase, como observado nas análises de cromatografia de permeação em gel e eletroforese em gel de poliacrilamida. Os peptídeos de caseína obtidos com a Flavourzyme também apresentaram melhores resultados utilizando o método ABTS na determinação da capacidade antioxidante. O hidrolisado obtido a partir da enzima Flavourzyme foi aplicado em produtos cárneos e em chocolate branco. Em produtos cárneos, os peptídeos de caseína (2.0%) inibiram, efetivamente, a peroxidação lipídica em carne moída (100%) e em carne mecanicamente separada de ave (CMS) (cerca de 20%) indicando que estes peptídeos podem ser utilizados nestes produtos, auxiliando na prevenção da formação de flavor desagradável e aumentando sua vida útil. Relativamente a sua aplicação em chocolate branco, esta adição teve o intuito de inibir escurecimento deste produto, fator considerado como limitante na sua vida-útil sendo conseqüência tanto de reações de escurecimento não enzimático quanto da oxidação de lipídeos. Os parâmetros que indicaram alteração lipidica e reações não enzimáticas foram mensurados em três diferentes amostras de chocolate branco: uma amostra com 0,2%, de manteiga de cacau, de antioxidante sintético Grindox 562, outra com 0,2%, de manteiga de cacau, dos peptídeos de caseína e a terceira amostra sem qualquer tipo de antioxidante. As amostras foram expostas a duas temperaturas diferentes: 20 ± 2 e 28 ± 2ºC. Os resultados das análises realizadas indicaram que as amostras armazenadas à temperatura de 20ºC apresentaram resultados significativamente melhores àqueles das amostras armazenadas à temperatura de 28ºC, relativos ao índice de acidez, à atividade de água, ao índice de peróxido, à cor e às substâncias reativas ao ácido tiobarbitúrico (TBARS), indicando melhor conservação deste produto. Também foi observado que a adição de quaisquer dos antioxidantes empregados não influenciou de forma significativa os resultados obtidos, evidenciando-se assim, que o principal parâmetro responsável pelas alterações do chocolate branco

em sua vida útil refere-se à temperatura de armazenamento a qual as amostras foram submetidas.

Palavras-chave: Caseína, Peptídeos de caseína, Hidrolise enzimática, Antioxidantes, Carne moída, Carne mecanicamente separada, Chocolate branco, Oxidação lipidica, Reação de Maillard

ABSTRACT

Recent studies indicate that peptides obtained by casein hydrolysis may have antioxidant activity. In this work, previous casein peptides were obtained by enzymatic hydrolysis using Alcalase and Flavourzyme (4h, at 50ºC and pH 8), selecting the ones that showed the best characteristics in vitro, related to the antioxidant activity. The enzymatic hydrolysis using Flavourzyme showed the best results, with higher soluble protein and free amino acid content and producing lower molecular weight peptides than Alcalase, as observed by gel permeation chromatography and polyacrylamide gel electrophoresis. Related to its application in meat products, casein peptides obtained with Flavourzyme also exhibited greater antioxidant capacity using the ABTS method. The casein hydrolyzed from Flavourzyme enzyme was applicated in ground beef homogenates, mechanically deboned meat (MDM) of poultry and white chocolate. In meat products, casein peptides (2.0%) effectively inhibited lipid peroxidation in ground beef homogenates (100%) and mechanically deboned meat (about 20%) of poultry. Casein peptides may be useful in meat processing as another naturally occurring antioxidant, helping to prevent off-flavor formation in meat products and increasing shelf life. In the use for white chocolate, the goal was to inhibit its browning, the main problems that limit the white chocolate's shelf-life. Non-enzymatic browning reaction and lipid oxidation were involved directly in the browning of white chocolate. Thus, parameters which indicated fat alteration and non-enzymatic reactions were measured in three different samples of white chocolate. One sample with 0,2% of cocoa butter, with the synthetic antioxidant Grindox 562, other with 0,2% of cocoa butter, with the natural antioxidant and the third sample without any kind of antioxidant. The samples were exposed to two different temperatures: 20 ± 2 and 28 ± 2ºC. The results of the analysis made indicated that the samples stored at the temperature of 20ºC showed results significantly better to those samples stored at the temperature of 28ºC, related to the conservation of the white chocolate. Besides, the results indicated that the addition of any antioxidants employees has not influenced in a significant way the results obtained. Thus, it was evidenced that the main responsible parameter for the alterations of the white chocolate's shelf-life is related to the storage temperature to which the samples were submitted.

Keywords: Casein peptides; Enzymatic hydrolysis; Antioxidant; Ground beef; MDM, White chocolate; Lipid oxidation; Maillard reaction.

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1 INTRODUÇÃO

1.1 Chocolate

Segundo dados da Associação Brasileira das Indústrias de Cacau, Amendoim e Balas (ABICAB), este setor teve em 2005, no Brasil, um faturamento de R$ 8,5 bilhões. Destes, aproximadamente 66% são representados pelos chocolates. Apresentou ainda, um acréscimo de 12,5% no consumo aparente e 8,5% na produção de chocolates no ano de 2006 em relação ao ano de 2005, segundo dados desta mesma associação.

Conforme o texto legal da Agência Nacional de Vigilância Sanitária (Anvisa), RDC Nº 264, DE 22 DE SETEMBRO DE 2005, o chocolate é o produto obtido a partir da mistura de derivados de cacau, massa (ou pasta ou liquor) de cacau, cacau em pó e ou manteiga de cacau, com outros ingredientes, contendo, no mínimo, 25 % (g/100 g) de sólidos totais de cacau. O produto pode apresentar recheio, cobertura, formato e consistência variados. O chocolate branco é o produto obtido a partir da mistura de manteiga de cacau com outros ingredientes, contendo, no mínimo, 20 % (g/100 g) de sólidos totais de manteiga de cacau. O produto pode apresentar recheio, cobertura, formato e consistência variados.

Na definição de Vercet (2002), o chocolate branco é o alimento composto de açúcar, sólidos de leite (leite integral em pó, mas algumas formulações podem conter leite desnatado ou lactose), manteiga de cacau, lecitina de soja e vanilina. Em substitutos de chocolate, a manteiga de cacau é substituída por outra gordura. Alander et al. (2002) acreditaram que o tipo de gordura utilizada na fabricação do chocolate implica, principalmente, nas características sensoriais, no valor de mercado, no tempo de prateleira e na finalidade do produto.

No entendimento de Beckett (1988), o chocolate tem duas características fundamentais: seu sabor e sua textura. Ainda que existam muitos sabores diferentes de chocolate, todos devem ser livres de sabores e odores desagradáveis. Uma particularidade básica da textura é que o chocolate deve ser sólido à temperatura ambiente de 20-25°C, porém fundir-se rapidamente na boca a 37°C.

Como referido anteriormente, na constituição do chocolate branco, encontram-se pequenas partículas de açúcar e de sólidos de leite que durante o processamento são cobertas pela gordura, seja manteiga de cacau ou um substituto desta. Por não conter

cacau em pó e massa de cacau, ingredientes importantes por suas características antioxidantes, são mais suscetíveis à oxidação de lipídeos quando comparado aos demais chocolates. Além disso, a cor escura que estes ingredientes conferem ao chocolate pode mascarar o escurecimento dos sólidos do leite como conseqüência de reações de escurecimento não-enzimáticas (VERCET, 2002).

Destarte, visando atender as exigências por parte dos consumidores, no sentido de fornecer produtos com qualidade até à hora de consumir tanto no que diz respeito à qualidade microbiológica dos produtos quanto na permanência de suas características sensoriais (KILCAST et al., 2000) objetiva-se a prevenção das reações escurecimento não-enzimáticas. De forma mais especifica cita-se a oxidação lipídica, em alimentos com percentual lipídico elevado, onde a utilização de antioxidantes se dá de maneira bastante extensa, e a reação de Maillard, na qual com seu retardamento está voltado ao controle de fatores extrínsecos durante o período de armazenamento.

1.2 Produtos Cárneos

Produtos cárneos são alimentos que, além de fornecerem uma matriz de nutrientes adequados para a proliferação de microorganismos, favorecem o desenvolvimento de uma série de reações indesejáveis ao produto final (AYMERICH et al., 2008).

Em carnes, a principal classe de lipídios no tecido adiposo (> 90%) é triacilglicerol. No tecido muscular, uma proporção significativa é fosfolipídica, que possui um teor elevado de ácidos graxos poliinsaturados e desempenham a sua função como um constituinte de celulares membranas (WOOD et. al., 2008).

Alimentos funcionais a base de carne estão tornando-se cada vez mais comuns e populares. Uma das principais fontes de matéria-prima para estes produtos refere-se à carne mecanicamente separada (CMS), sendo justificada pelo seu valor econômico (PÜSSA et. al., 2008). No Brasil, torna-se ainda mais relevante a utilização de CMS pois sabe-se que este país é o terceiro maior produtor de carne de frango do mundo com uma produção total de 6,5 Milhões de toneladas em 2001 (APA, 2002). É calculado que pelo menos 20% das carcaças frescas de frangos de corte são transformados em carne de frango mecanicamente separada (CMS). Desta forma, cerca de 1,3 milhões de toneladas de CMS foram produzidos no Brasil, em 2001. A legislação brasileira permite

um máximo de 20% do total de carne fresca possa ser substituído por CMS (Brasil, 1981). Ossos, cartilagens, pele e tecidos estão normalmente presentes e a composição química do CMS varia, dependendo da origem da matéria-prima, ou seja, aumentar tecidos da pele aumenta a fração lipídica e diminui a fração proteíca (NEGRÃO et al., 2005). Os principais problemas relativos ao consumo deste produto referem-se à intoxicação tanto bacteriana quanto a doses potencialmente mutagênicas e cancerígenas proveniente da oxidação de ácidos graxos ingeridas. O oxigênio, que é vinculado à massa de carne e enzimas, bem como heme liberado devido a aeração durante o estresse mecânico de moagem catalisam a peroxidação de ácidos graxos poliinsaturados (PUFA) e, conseqüentemente, aceleram a deterioração oxidativa do CMS (PÜSSA et. al., 2008).

Além disso, a composição dos ácidos graxos também afeta a estabilidade dos produtos cárneos relativamente a degradação oxidativa durante o processamento. Um teste padrão para medir a estabilidade do processo oxidativo de lipídios em alimentos é o teste de substâncias reativas ao ácido tiobarbitúrico (TBARS), que mede a oxidação lipídica através da formação do produto malondialdeído. Valores de malonaldeído acima de 0,5 são considerados críticos, uma vez que indicam um nível de produtos da oxidação lipídica que produzem odor e gosto a ranço que podem ser detectadas pelos consumidores (Wood et. al., 2008). Assim, a oxidação lipídica é a maior causa de perda de aroma e valor nutritivo de produtos contendo gordura. Com vistas a sanar este problema, antioxidantes sintéticos como o butil-hidroxil tolueno (BHT) e butil-hidroxil anisolo (BHA) têm sido amplamente utilizados na indústria alimentícia. Contudo, a evidência de toxicidade destes produtos tem proporcionado o impulso para a busca de alternativas antioxidantes (HASSAN et al., 2005) que reagem com elétrons oxidantes, resultando no seqüestro de radicais livres e complexação de metais prooxidantes (HASSAN et al., 2005).

1.3 Reações de Escurecimento Não-enzimático

1.3.1 Reação de Maillard

Em 1912, Louis Camille Maillard trabalhando em Sorbonne, Paris, observou o processo de escurecimento na mistura de glicose-glicina e suspeitou do grande impacto deste processo na química orgânica, ciência de alimentos, biologia e geologia. Devido a

esta suspeita, pesquisas sobre este tema avançaram durante todo século passado gerando dados significativos confirmando às predições originais do autor (AJANDOUZ et al., 2008).

Este processo de escurecimento, o qual ficou conhecido como reação de Maillard, é uma complexa série de reações químicas que ocorrem entre carbonilas, especialmente açúcares redutores, e compostos com grupos amino livres, como aminoácidos e proteínas. É classificada como uma reação de escurecimento não-enzimático (JALBOUT et al., 2007) e pode ser visualizada, de forma geral, através da Figura 1.

A reação de Maillard é de máxima importância para a qualidade dos alimentos. Além de induzir o escurecimento nos alimentos, tem efeito no valor nutritivo, pode provocar implicações toxicológicas (como a formação de acrilamida), produzir compostos com ação antinutricional e antioxidante, e também influenciar na formação do flavor (BOEKEL, 2006).

Em se tratando de chocolate branco, considerando o significativo percentual de sólidos de leite em sua composição, estes são os responsáveis pelo desenvolvimento da reação de Maillard, onde o açúcar redutor é a lactose, um dissacarídeo formado por glicose e galactose, encontrado no leite em concentrações de 4.5 a 5.0 g/100mL (MESSIA et al., 2007) e o grupamento amino é composto, principalmente, por resíduos de lisina. Deve-se esclarecer que (o conteúdo de aminoácidos livres do leite é baixo) (BOEKEL, 1998).

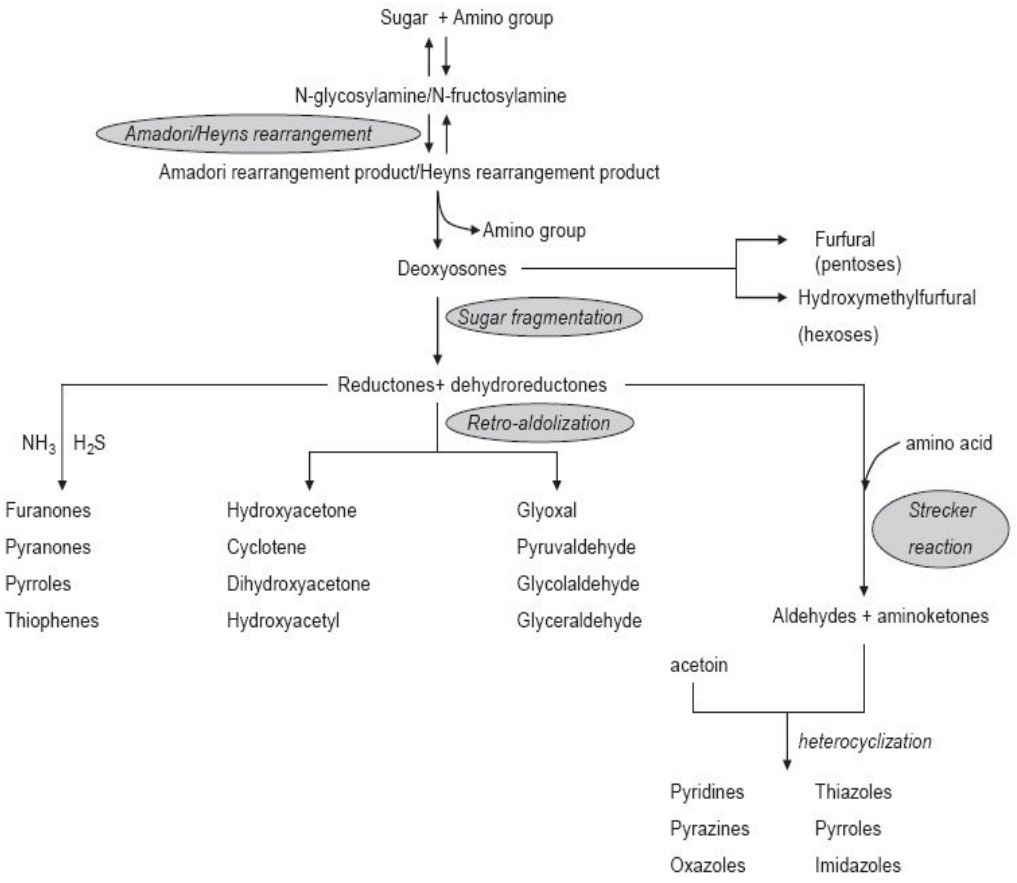

Fig. 1: Visão geral da reação de Maillard, ilustrando a formação de compostos saborizantes como produtos finais (Boekel, 2006).

Grande parte da literatura considera a reação de Maillard como uma série de reações subseqüentes e paralelas, que podem estar divididas em três estágios: o estágio inicial, o avançado e o final (SILVÁN et al., 2006), podendo ser influenciada por fatores tais como: tempo, temperatura, pH, atividade de água (aw), natureza e concentração dos reagentes (JING et al., 2002).

O estágio inicial da reação de Maillard consiste na condensação do açúcar redutor com o grupo amino, via formação da base de Schiff e o rearranjo de Amadori, resultando nos compostos de Amadori. No leite, este composto de Amadori é a lactulolisina (BOEKEL, 1998). Compostos de Amadori são precursores de numerosos produtos reconhecidos como flavorizantes característicos, aromas e polímeros escuros. Eles também são responsáveis pela perda de valor nutricional de aminoácidos e proteínas, como conseqüência da redução da disponibilidade biológica destes nutrientes por envolvimento na formação dos compostos de Amadori (SILVÁN et al., 2006).

O estágio avançado da reação de Maillard inicia com a quebra dos compostos de Amadori, conduzindo a produtos de numerosas fissões do açúcar e liberando grupos amino (BOEKEL, 2006). Existem três rotas gerais para a quebra destes compostos: a rota das 3-desoxiosonas via 1,2 enolização em pH ácido; a rota das 1-desoxiosonas via 2,3 enolização, favorecida sob pH neutro ou alcalino (DATTATREYA et al., 2007); enquanto que a terceira rota, a rota 4-desoxiosona é significante para dissacarídeos em condições estritamente alcalinas (BOEKEL, 1998).

No pH do leite (pH 6,6), a degradação dos compostos de Amadori pode ser obtida, principalmente, pela a rota da 2,3 enolização. Dissacarídeos, como a lactose, diferem em sua rota dos monossacarídeos, como a glicose, devido à ligação 1,4 glicosídica (BOEKEL, 1998).

O estágio final da reação de Maillard conduz para todos os tipos de reações de desidratação, fragmentação, ciclização e polimerização onde os grupos amino participam (BOEKEL, 2006). Neste estagio, ocorre a formação de pigmentos escuros (melanoidinas) provenientes de compostos reativos formados no estagio avançado e onde ocorre ligações cruzadas da proteína. No caso do leite, os componentes escuros são provenientes principalmente ligações protéicas. Embora a natureza destas ligações não seja clara, acredita-se que os resíduos de lisina estejam envolvidos na formação de melanoidinas, haja vista que há um aumento na perda de lisina durante o estagio final (BOEKEL, 1998; WEENEN, 1998).

1.3.2 Oxidação Lipídica

Óleos e gorduras são usados em alimentos por muitas razões. São nutrientes, fontes de energia e de ácidos graxos essenciais e viabilizam outros nutrientes como as vitaminas lipossolúveis. São importantes, também, na textura e sabor dos alimentos influenciando na palatabilidade e aceitação do produto final (ALANDER et al., 2002).

As gorduras vegetais são provenientes de diversas árvores tropicais e se caracterizam por seu baixo ponto de fusão, devido principalmente a disposição dos ácidos graxos nas moléculas de triacilglicerol (WONG, 2003).

Os ácidos graxos tendem a se distribuir uniformemente entre todas as moléculas de triacilglicerol. Aproximadamente 80% dos triacilglicerois da manteiga de cacau contêm dois ácidos graxos insaturados e os ácidos graxos saturados nas posições primárias (WONG, 2003).

O principal problema deteriorativo com lipídeos está relacionado à oxidação. Os processos oxidativos degradam lipídeos e proteínas (incluindo pigmentos) e são mecanismos principais de perda de qualidade de produtos alimentícios (HAAK et al., 2006). A oxidação de lipídeos é uma reação em cadeia que afeta os ácidos graxos poliinsaturados através da ação de radicais livres (CASCONE et al., 2006).

A oxidação lipídica é um fenômeno complexo induzido pelo oxigênio na presença de iniciadores como temperatura, radicais livres, pigmentos fotossensíveis e íons metálicos (LAGUERRE et al., 2007). Ela acontece durante o armazenamento e processamento de alimentos e possuem ligação com o desenvolvimento de rancidez e sabor desagradável, assim como um potencial de reações tóxicas nos produtos. O controle da oxidação de lipídeos nos alimentos é desejável e os benefícios dos antioxidantes no armazenamento de alimentos têm sido cada vez mais estudados (SAKANAKA et al., 2005).

Três são as possíveis rotas de desenvolvimento da oxidação de lipídeos: cadeia de autooxidação não-enzimática mediada por radicais livres, fotooxidação não-enzimática e não-radical e a oxidação enzimática. Os primeiros dois tipos de oxidação consistem na combinação de reações envolvendo oxigênio tripleto, e o oxigênio singleto que corresponde ao estado excitado da molécula (LAGUERRE et al., 2007).

A estabilidade da oxidação de gorduras vegetais é determinada por seus graus de insaturação, presença de antioxidantes naturais ou sintéticos, de pró-oxidantes como metais, disponibilidade de oxigênio, temperatura e luz. A oxidação de triacilglicerois normalmente acontece no lugar das duplas ligações e formam hidroperóxidos (produto da oxidação primária), cetonas e aldeídos (produtos da oxidação secundária) (KRISTOTT, 2000).

Antioxidantes sintéticos são largamente utilizados em produtos alimentícios para retardar a oxidação de lipídeos, porém, a demanda por antioxidantes naturais tem aumentado por causa da toxicidade e carcinogenicidade dos antioxidantes sintéticos (SAKANAKA et al., 2005).

Atualmente há uma forte tendência no isolamento de antioxidantes orgânicos de fontes naturais como forma alternativa na proteção dos produtos contra a oxidação (HAAK et al., 2006). Muitos antioxidantes são substâncias que foram isoladas de materiais naturais, inclusive alimentos. Dentre esses, aminoácidos e proteínas tem sido relatados por serem solúveis em água e possuir o efeito de complexar íons metálicos.

Além disso, algumas proteínas hidrolisadas de animais e plantas possuem atividades antioxidantes (SAKANAKA et al., 2005).

Estudos recentes mostram que a caseína, proteína majoritária do leite, quando hidrolisada possui propriedades antioxidantes. Os hidrolisados de caseína, além de serem ingredientes versáteis em alimentos, possuem propriedades funcionais e nutricionais (KITTS, 2005).

1.4 Hidrolisado de Caseína

O leite e produtos lácteos têm sido usados, comumente, para realçar a qualidade nutricionai e tecnológica de diferentes alimentos bem como adicionar a eles sabor desejado. Entre as causas subjacentes de tais aplicações pode-se citar a alta qualidade nos valores nutritivos de produtos lácteos e a distribuição homogênea destes na composição do alimento no qual são adicionados (TUNÇTÜRK e ZORBA et al., 2006). As atividades biológicas e fisiológicas das proteínas do leite são atribuídas parcialmente a diversos peptídeos presentes nas moléculas hidrolisadas da proteína (KIM et al., 2007).

A hidrólise das proteínas pode ser feita através de enzimas *in vivo* ou *in vitro*. Os hidrolisados da proteína podem ser classificados em três grupos principais dependendo do grau de hidrólise, isso determina suas aplicações: hidrolisados com um baixo grau de hidrólise são usados para melhorar propriedades funcionais, hidrolisados com um grau variável de hidrólise são usados na maior parte como saborizantes, e hidrolisados com alto grau de hidrólise são usados na maior parte como suplementos nutritivos e em dietas médicas especiais (PEDROCHE et al., 2004).

Os hidrolisados de caseína obtidos por proteases como tripsina e quimiotripsina contêm mais de 200 peptídeos (SAKANAKA et al., 2005) com massa molecular menor e a estrutura secundária diferente das proteínas intactas (FitzGerald et al., 1998) o que faz com que sejam melhor absorvidos (SAKANAKA et al., 2005).

A caseína quando hidrolisada possui moléculas biologicamente ativas, entre elas peptídeos fosforilados, que exercem efeito na biodisponibilidade do cálcio e também em outros minerais por causa do caráter altamente aniônico (FITZGERALD, 1998; KITTS, 2005).

É conhecida a habilidade do hidrolisado de caseína de seqüestrar radicais livres bem como complexar metais de transição tais como o cálcio, o ferro, o cobre, e o zinco

(KIM et al., 2007). A complexação de metais da transição pelos peptídeos de caseína podem ser originados pelos resíduos de fosfoseril e glutamil contidos na α-, ß- e κ-caseína (MEISEL, 1997) que diferem entre si pelo seu conteúdo de fosfato (10, 5, e 1 mol de caseína, respectivamente) (SAKANAKA et al., 2005). A capacidade dos peptídeos de seqüestrar radicais livres tem sido correlacionada positivamente com a quantidade de histidina, de lisina, de prolina e de tirosina, que contribuem com a atividade antioxidante de algumas proteínas e peptídeos (KIM et al., 2007). Diante das características expostas inúmeros pesquisadores, entre eles, Sakanaka et al. (2005), sugerem que estes peptídeos podem ser usados, de maneira eficaz, como antioxidantes, evitando danos oxidativos em alimentos e aumentando a vida útil dos mesmos (SAKANAKA et al., 2005).

1.5 Objetivo Geral

Diante do exposto, o presente trabalho objetiva-se obter um antioxidante natural proveniente da hidrólise enzimática da caseína. Além disso, pretende-se caracterizar e quantificar este antioxidante natural *in vitro,* e, investigar seu efeito inibitório frente a oxidação lipídica em sistemas alimentícios tais como, carne moída, carne mecanicamente separada de ave e chocolate branco.

A seguir, são apresentados os resultados da presente investigação relatados na forma de dois artigos científicos que expressam tanto as etapas de produção e caracterização dos peptídeos de caseína e seus efeitos na oxidação lipídica em carne moída e carne mecanicamente separada de ave, quanto a quantificação de produtos presentes ou gerados ao longo da vida útil de diferentes formulações de chocolate branco quando exposto a diferentes temperaturas demonstrando os resultados e as conclusões obtidas no desenvolvimento deste projeto.

2 RESULTADOS

Os resultados deste trabalho estão apresentados na forma de artigos a serem submetidos para publicação nas revistas Food Chemistry e Journal of Food Composition and Analysis. Cada subtítulo deste capítulo corresponde a um destes artigos.

2.1 Production and characterization of casein peptides with antioxidants properties and their effects on lipid oxidation in beef homogenates and MDM of poultry.

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Abstract

Recent studies indicate that peptides obtained by casein hydrolysis may have antioxidant activity. In this work, casein peptides were obtained by enzymatic hydrolysis using Alcalase and Flavourzyme (4h, at 50ºC and pH 8). The enzymatic hydrolysis using Flavourzyme showed the best results, with higher soluble protein and free amino acid content and producing lower molecular weight peptides than Alcalase, as observed by gel permeation chromatography and polyacrylamide gel electrophoresis. Casein peptides obtained with Flavourzyme also exhibited greater antioxidant capacity using the ABTS method. Casein peptides (2.0%) effectively inhibited lipid peroxidation in ground beef homogenates (100 %) and mechanically deboned meat of poultry (about 20%). Casein peptides may be useful in meat processing as another naturally occurring antioxidant, helping to prevent off-flavor formation in meat products and increasing shelf life.

Keywords: Casein peptides; Enzymatic hydrolysis; Antioxidant; Ground beef; MDM.

Introduction

Milk and milk products have been frequently used in the enhancement of nutritional and technological qualities of a wide range of different foods, as well as in addition the desired flavor to them (Tunçtürk et al., 2006). The biological and physiological activities of milk proteins are partially attributed to several peptides encoded in the native protein molecules (Kim et al., 2007). They can be produced *in vitro* by enzymatic hydrolysis.

The enzymatic hydrolysis of proteins has been extensively used to produce food ingredients with improved functional and nutritional properties (Lemieuxa et al., 1997). Protein hydrolysates can be classified into three major groups depending on the degree of hydrolysis, which determines their applications: hydrolysates with a low degree of hydrolysis, with improved functional properties, hydrolysates with a variable degree of hydrolysis, that are mostly used as flavorings, and extensive hydrolysates, that are mostly used as nutritional supplements and in special medical diets (PEDROCHE et al., 2004).

Casein hydrolysates obtained by the proteases trypsin and chymotrypsin contain more than 200 peptides of different sizes (Sakanaka et al., 2005) and have smaller molecular masses and less secondary structure than intact proteins (Fitzgerald, 1998). The hydrolysates are better absorbed than a mixture of free amino acids, which may be due to the size and nature of the peptides during the digestive process (Sakanaka et al., 2005). Among the biologically active peptide molecules, the phosphorylated caseinophosphopeptides (CPP), are known to exert an effect on calcium bioavailability but also on other minerals because of the highly anionic character of CPP which makes them resistant to further hydrolysis by proteases and allow them to form soluble complexes with calcium (FITZGERALD, 1998; KITTS, 2005).

The ability of CPP to scavenge free peroxyl radicals as well as to chelate the transition metals such as calcium, iron, copper, and zinc has been reported (Kim et al., 2007). The chelation of transition metals by CPP might originate from phosphoseryl and glutamyl residues contained in α-, ß-, and κ-casein (Meisel, 1997) which differ from each other in their phosphate content (Sakanaka et al., 2005). The scavenging capacity of CPP against free peroxyl radicals has been demonstrated to be positively correlated with the amounts of histidine, lysine, proline,

and tyrosine that might contribute to the antioxidant activities of some proteins and peptides (Kim et al., 2007). It has been suggested that CPP could be used as antioxidants to prevent oxidative damage to muscle foods (SAKANAKA et al., 2005).

Rancidity of food of lipid oxidation is a serious problem because it not only produces off-flavors but also decreases the nutritional quality and safety of the food (Shih et al., 2003). Therefore, the control of lipid oxidation in food products is desirable, and the benefits of antioxidants in food storage have been studied by many researchers (Sakanaka et al., 2005). Synthetic antioxidants, such as butylated hydroxylanisole (BHA), butylated hydroxytoluene (BHT), propyl gallate (PG), and tert-butylhydroquinone (TBHQ), have been commonly used to suppress the formation of free radicals, preventing lipids from oxidation and food spoilage. Although these synthetic reagents are efficient and relatively cheap, special attention has been given to natural antioxidants because of a worldwide trend to avoid or minimize the use of synthetic food additives (SHIH and Daigle, 2003).

The aims of this study were to characterize casein peptides obtained by casein hydrolysis with commercial proteases, to measure the antioxidant activity of casein peptides and to investigate the inhibitory effect of casein peptides on lipid oxidation in ground beef homogenates and mechanically deboned meat of poultry (MDM).

2. Materials and Methods

2.1 Reagents

Trinitrobenzenezulfonic acid, glycine, bovine serum albumin (BSA), leucine, HEPES (2-4-2-hydroxy-ethyl-1-piperazinyl ethanesulfonic acid) and 2-thiobarbituric acid were obtained from Sigma (St. Louis, MO, USA). Casein was from Farmaquimica (São Paulo, Brazil). Ninhidrin was from Merck (Darmstadt, Germany). Sodium docecyl sulfate (SDS) and trichloroacetic acid were from Nuclear (Rio de Janeiro, Brazil). Alcalase® (endoproteinase from Bacillus licheniformis) and Flavourzyme™ (endoprotease and exopeptidase from Aspergillus oryzae) were from Novozymes

Latin America (Bento Gonçalves, Brazil). Commercial antioxidant Grindox 562 was from Danisco (Copenhagen, Denmark). MDM was from Doux-Frangosul (Montenegro, Brazil) and ground beef was purchased from a local supermarket (Porto Alegre, Brazil).

2.2 Hydrolysis of casein

Casein peptides were prepared by the hydrolysis of casein using the proteolytic enzymes Alcalase and Flavourzyme. Casein was dissolved in distilled water pH 8.0 at a concentration of 130 g l-1 and hydrolyzed by protease (0.4/100 enzyme/substrate ratio) at 50ºC in a stirred bath. During the reaction, aliquots were taken out at various time intervals (between zero and 240 min). The hydrolysis was stopped by adding trichloroacetic acid (TCA) to reach a final concentration of 10% (w/v). Hydrolysates were centrifuged at 10,000 x g for 20 min to remove insoluble materials. The hydrolysates were then frozen, lyophilized and kept at -18ºC before further analysis.

2.3 Determination of soluble protein concentration

The concentration of soluble protein was determinate by the Folin phenol reagent method (Lowry et al., 1951). Bovine serum albumin (BSA) was used as standard. The measurements were performed using a spectrophotometer UV mini - 1240 Shimadzu (Shimadzu do Brasil, Agua Branca, SP, Brazil).

2.4 Determination of amino acid concentration

Concentration of amino acids was determined by the ninhidrin method (Moore and Stein, 1957). Glycine was used as standard. The measurements were performed using a spectrophotometer UV mini - 1240 Shimadzu (Shimadzu do Brasil, Agua Branca, SP, Brazil).

2.5 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

Casein hydrolysates were analyzed by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE) as described by LaemmLi (1970), using a 14% acrylamide resolving gel and a 4% acrylamide stacking gel. Electrophoresis of the protein samples, dissolved in the sample buffer (4% SDS, 20% glycerol, 10% ßmercaptoethanol, 0.125 M Tris-HCl, pH 6.8), was run with an SE 250 Mighty Small II slab gel electrophoresis unit (Hoefer Scientific Instruments, San Francisco, CA, USA). Aliquots of 15µL of casein hydrolysate per well were loaded onto the gel. The relative molecular mass of the peptides was determined by comparing with known standard molecular mass markers (10–170 kDa; Fermentas Inc., Canada). After electrophoresis, the protein was detected with silver staining (Switzer et al., 1979).

2.6 Gel permeation chromatography

The chromatographic analysis of the casein hydrolysates was performed by gel filtration on a Biogel P6-DG column (0.9 x 18 cm) eluted with 10 mM phosphate buffer pH 7.4. Fractions of 1 mL were collected and evaluated for protein concentration with ultraviolet absorption at 280 nm.

2.7 ABTS radical antioxidant activity

The antioxidant activity was determined using ABTS•+ (2,2-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)) radical cation as described by Re et al. (1998). ABTS was dissolved in water to 7 mM concentration. ABTS radical cation (ABTS•+) was produced by reacting ABTS stock solution with 2.45 mM potassium persulfate (final concentration) and allowing the mixture to stand in the dark at 25±2ºC temperature for 16 h before using. Stock solution was used for a maximum of 3 days. In the moment of use, the ABTS•+ solution was diluted with PBS, pH 7.4, to an absorbance of 0.70 ± 0.02 at 734 nm. Samples were diluted to obtain between 20–80% inhibition of the blank absorbance. Ascorbic acid was used as standard

(0 - 9 µg). After addition of 10 µL of sample (or standards) in 2.0 mL of diluted ABTS•+ solution, the absorbance reading was followed during 5 min. Appropriate solvent blank was run in each assay. All determinations were carried out at least three times. The percentage inhibition of absorbance at 734 nm is calculated and plotted as a function of concentration of antioxidants and ascorbic acid for the standard reference data.

2.8 Amino acid composition

Determination of amino acid composition of the ground samples of casein and hydrolyzed casein was performed at Faculdade de Medicina de Ribeirão Preto (UNESP, Ribeirão Preto, Brazil). Amino acid analysis was performed by ion-exchange chromatography (Spackman et al, 1958). The amino acids were obtained by peptide hydrolysis with 6 M HCl at 110 ± 1 ºC for 22 h and purified with Amberlite IR-120. Tryptophan was determined separately after hydrolysis was carried out in 4M LiOH at 110 ± 1°C for 24 h as described by Penke et al. (1974).

2.9 Antioxidant activity in ground beef homogenates and MDM

Antioxidant activity in ground beef homogenates and MDM was determined as described Sakanaka et al. (2005). Ground beef (containing 13.2% fat) and MDM (containing 20.65% fat) were used in this experiment. Ground beef (5g) and MDM was homogenized in 25 mL of 50 mM HEPES buffer (pH 7.0). Buffered systems have been widely used to study oxidation-reductions in meat systems. The test medium contained 0.8 mL of beef homogenate and 0.2 mL of either the HEPES buffer or one of the sample solutions (casein peptides in HEPES buffer) to give a final concentration of 0.25, 0.5, 1.0, 2.0 and 4.0% of casein peptides and was incubated at 37ºC for 60 min. After incubation, the mixture was tested for the formation of thiobarbituric acid reactive substances (TBARS). On day of use, a trichloroacetic acid (TCA/TBA) stock solution was prepared consisting of 15% TCA (w/v) and 0.375% TBA (w/v) in 0.25 M HCl. After mild heating and agitation to dissolve the components, 3 mL of 2% butylated hydroxytoluene (BHT) in absolute

ethanol was added per 100 mL of the TCA/TBA solution stock. At appropriate intervals, 1.0 mL of aliquot of the test medium was added to the TCA/TBA stock solution in a test tube and immediately mixed. The sample was then heated in a boiling water bath for 10 min and cooled to room temperature, and it was centrifuged at 1710 x g for 10 min. The absorbance of the supernatant was measured at 532 nm. TBARS were calculated from a standard curve of malonaldehyde (MDA), a breakdown product of tetraethoxypropane (TEP).

3. Results

The enzymatic hydrolysis of casein with Alcalase and Flavourzyme was monitored for up to 4h. Data. In Figure 1A shows the curve obtained under experimental conditions, resulting in an increase of concentration of soluble protein as a function of reaction time with both enzymes. Enzymatic hydrolysis with Flavourzyme resulted in more soluble protein release, in agreement with amino acids content showed in Figure 1B.

The Figure 2 shows the gel permeation chromatography profiles of casein and casein hydrolyzed with both enzymes. It was found that the Flavourzyme and Alcalase were effective in the breaking down of the native casein molecules. The casein hydrolyzed by Flavourzyme showed a molecular weight distribution corresponding to smaller values compared with Alcalase. Other technique relying on the determination of the molecular weight profile of casein hydrolysates and to confirm hydrolysis was achieved by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE). Electrophoretic patterns of the hydrolysates obtained showed the enzyme treatments were remarkably effective in degrading casein, ostensibly into small peptides and amino acids with Flavourzyme enzyme, which showed presence of smaller molecular weight bands (Figure 3).

Casein peptides obtained by enzymatic hydrolysis exhibit direct free radical scavenging activity in aqueous medium, as shown using the pre-formed stable ABTS radical (Kitts, 2005). Antioxidant activity of casein hydrolyzed with Flavourzyme presented values of 23.9 ± 2.3 compared with 7.7 ± 0.8 for Alcalase, expressed as mg Eq vitamin C mL-1. Value obtained for a commercial antioxidant (Grindox 562) was 37.5 ± 1.9. As the results obtained with Flavourzyme

were better than those obtained for Alcalase, next experiments were carried out only for Flavourzyme hydrolysate. The quenching affinity for casein peptides towards the ABTS radical was concentration dependent *in vitro* (Figure 4). Maximum activity accounting for 99.5% of the radicals was observed at 100 mg mL-1, but about 90% inhibition was obtained with 10 mg mL-1.

The amino acid composition of casein and casein peptides resulting from hydrolysis with Flavourzyme are presented in Table 1. The hydrolysate has a very similar amino acid profile to that observed for casein although it showed minor amount of Lys and Leu. However, when the amino acid composition of the hydrolysate was determined before acid hydrolysis (free amino acids only), a quite different composition was observed (Table 1). The amount of sulfur-containing amino acids Cys and Met was higher in NAOH, as well as the essential Lys, His, Leu e Ile. In contrast lower amounts of Asp and Glu, Trp and Ser were not detected.

As casein peptides, hydrolyzed with Flavourzyme enzyme, showed antioxidant activity with ABTS in vitro, their activity was tested using a meat model system. Casein peptides were added to the beef homogenates and MDM at different concentrations, and lipid oxidation was evaluated. The reaction was measured by monitoring TBARS, and the results are shows in Figures 5 and 6. When incorporated into meat products, casein peptides (2.0%) inhibit 100% of lipid oxidation in round beef homogenates and about 21% in MDM.

4. Discussion

In this work, casein peptides were obtained by enzymatic hydrolysis of casein using the food grade proteases Alcalase and Flavourzyme. Enzymatic hydrolysis with Flavourzyme resulted in increased soluble protein and free amino acids release. Results obtained in gel permeation chromatography showed that the Flavourzyme hydrolyzed the native casein molecules into smaller peptides than Alcalase. Electrophoretic patterns of casein and the hydrolysates confirm these results. Both enzymes have been described as good catalysts to produce food protein hydrolysates, although those produced with Alcalase can have a less bitter taste since it has broad specificity with some preference for terminal hydrophobic amino acids (Lahl and Braun, 1994). As small peptides are related with antioxidant activity (Kitts, 2005), data

obtained comparing antioxidant activity confirm this results and Flavourzyme was selected for further analysis.

Production of free amino acids by Flavourzyme could be observed by amino acid analysis, since the amino acid composition of casein peptides could be determined before treatment with hydrochloridric acid. The amino acid profile was quite distinct of those observed for casein, indicating that some amino acids were preferentially released. This result indicated that hydrolysis of casein causes no loss of the amino acids conserving its high nutritional value.

Short proteolysis has been used to modify the functional properties of proteins, such as solubility, gelation, and emulsification, and for development of hypoallergenic formula (Lahl and Braun, 1994). In addition, several studies have reported the importance of peptides from casein that have biological or technical functions (Clare and Swaisgood, 2000). The bioactivities of peptides encrypted in milk proteins are latent until activated by enzymatic proteolysis. Milk whey peptides obtained by hydrolysis with fungal protease showed anti-hypertensive activity (Sinha et al., 2007) and casein-derived peptides from cheese showed antimicrobial/antioxidant activity.

The quenching affinity of casein peptides towards the ABTS radical was concentration dependent, with a maximum activity accounting for 99.5% of the radicals at 100 mg mL-1. Similar results were obtained by Kitts (2005), with a maximum activity for 91.8% of the radicals at 1.0 mg mL-1 of casein calcium peptides (CCPs). The author suggest that CCPs may be useful in meat processing as other naturally occurring antioxidants, helping to prevent the formation of an off-flavor in meat products thereby increasing shelf life. Besides the presence of CCPs, the hydrolysate showed free Cys, which is an amino acid with known antioxidant activity.

Antioxidant activity was also tested using a meat model system. Beef homogenate and MDM may be effective media to investigate the protective effect of water-soluble antioxidants against lipid peroxidation (Lee & Hendricks, 1997). When incorporated into ground beef homogenates and MDM, casein peptides (2.0%) inhibits 100% of lipid oxidation in homogenates and about 21% in MDM. Sakanata et al. (2004) obtained 69.7% of inhibition in ground beef with 2.0% of casein calcium peptides (prepared by microbial enzyme hydrolysis of casein calcium)

and Lee & Hendricks (1997) obtained 76.2% of inhibition using 20 mM of carnosine (an endogenous dipeptide found in the skeletal muscle of most vertebrates).

5. Conclusion

Between the two enzymes tested, the enzymatic hydrolysis of casein through the action of the enzyme Flavourzyme enzyme showed the best results. Casein peptides hydrolyzed by Flavourzyme had higher soluble protein content and free amino acid content, indicating how enzyme might be more effective in producing peptides of low molecular weight than Alcalase. This fact was confirmed by gel permeation chromatography and electrophoretic patterns of the hydrolysates. Although, casein peptides obtained with Flavourzyme exhibited significantly greater antioxidant capacity, compared to casein peptides obtained with Alcalase. These results suggest that casein peptides are a good source of natural antioxidant. Casein peptides (2.0%) effectively inhibited the lipid peroxidation in ground beef homogenates (100 %) and MDM (about 21%). At present, consumer demand for natural functional foods has been increasing, and therefore casein peptides can be used as a functional food ingredient in pharmaceutical and food industries. Casein peptides may be useful in meat processing as another naturally occurring antioxidant, helping to prevent off-flavor formation of meat and its products and increasing shelf life.

Acknowledgements

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Table 1: Comparative amino acid composition of casein peptides and casein (%µmol/mg sample)

Amino Acid	Casein Peptides (1)	Casein (2)	Casein Peptides (2)
Thr	1.635±0.120	0.736±0.014	0.730±0.004
Lys	12.130±0.930	8.061±0.009	7.753±0.015
His	5.380±0.433	2.836±0.012	2.935±0.007
Arg	5.722±0.421	3.204±0.004	3.207±0.003
Asp	4.679±0.326	7.273±0.060	7.078±0.019
Trp	0.000	4.555±0.008	4.530±0.006
Ser	0.000	6.901±0.039	6.760±0.006
Glu	12.295±0.775	17.083±0.025	16.888±0.018
Pro	5.352±0.394	10.569±0.018	10.685±0.024
Gly	1.594±0.099	3.129±0.006	3.402±0.020
Ala	5.483±0.367	4.346±0.047	4.703±0.002
Cys	0.870±0.064	0.524±0.007	0.420±0.012
Val	9.886±0.691	6.651±0.039	7.403±0.021
Met	3.995±0.313	2.212±0.001	2.535±0.001
Ile	7.770±0.349	5.019±0.003	5.460±0.010
Leu	16.819±0.811	9.036±0.069	8.819±0.037
Tyr	4.536±0.370	3.939±0.001	2.990±0.038
Phe	6.804±0.540	3.923±0.002	3.701±0.019

(1) Without hydrolysis
(2) With hydrolysis: with 4N LiOH for tryptofane for 24h at 110°C ± 1°C and with 6N HCl for other amino acids at 110°C ± 1°C for 22h.

Figure Legends

Fig. 1. Soluble protein concentration (A) and amino acid concentration (B) using Flavourzyme™ (■); Alcalase® (♦) and control (▲).

Fig.2. Gel permeation chromatography (Biogel) of casein peptides using enzyme Flavourzyme™ (▲); Alcalase® (×) and control (■).

Fig.3. Electrophoretic patterns of the hydrolysates obtained from the enzyme treatments: (a) casein; (b) casein peptides obtained by Alcalase®; (c) casein peptides obtained by Flavourzyme™.

Fig.4. ABTS radical inhibition (%) of casein peptides obtained by Flavourzyme™ hydrolysis. Data represent the mean ± SD of at least three determinations.

Fig.5. Effect of casein peptides obtained by Flavourzyme™ hydrolysis on the formation of TBARS in ground beef homogenates. Data represent the mean ± SD of three determinations.

Fig.6. Effect of casein peptides obtained by Flavourzyme™ hydrolysis on the formation of TBARS in MDM. Data represent the mean ± SD of three determinations.

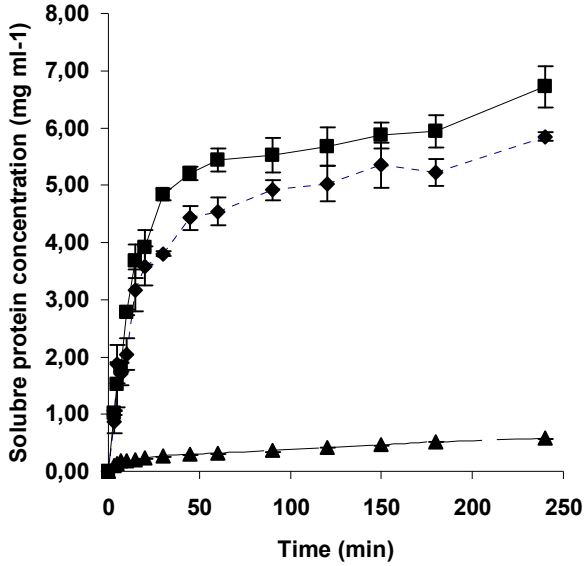


Fig.1A. Rossini et al., 2007

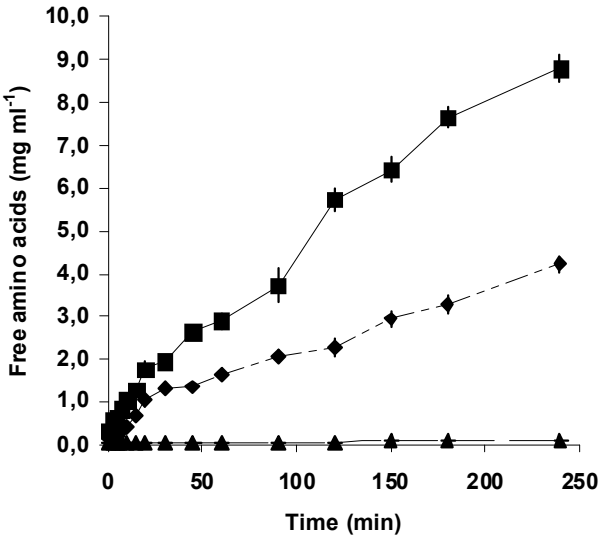


Fig.1B. Rossini et al., 2007

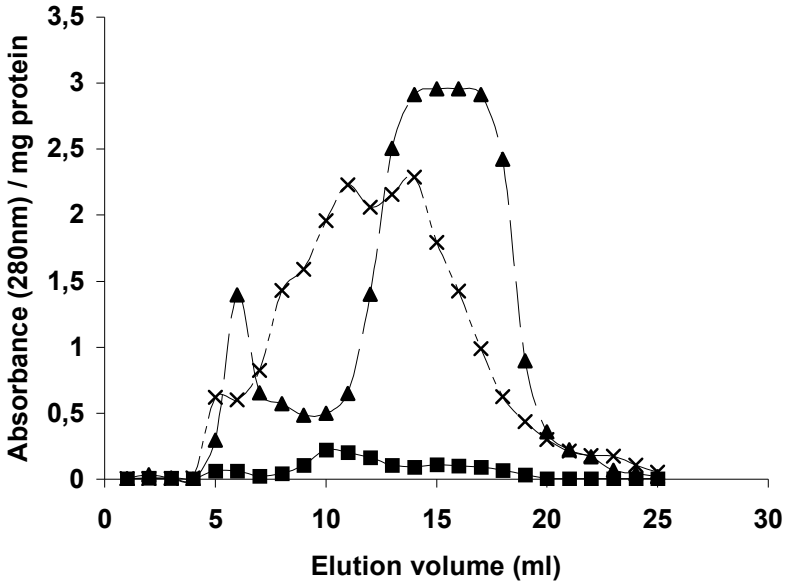
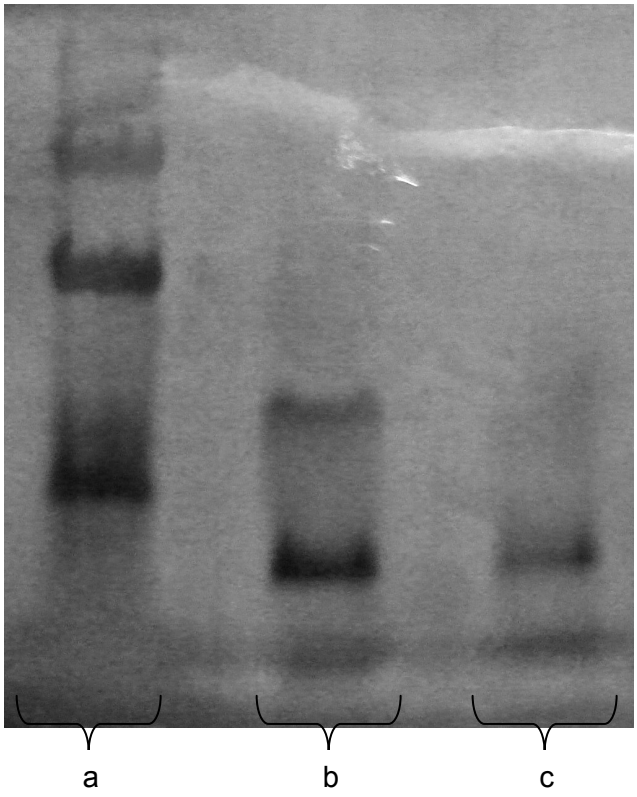


Fig.2. Rossini et al., 2007



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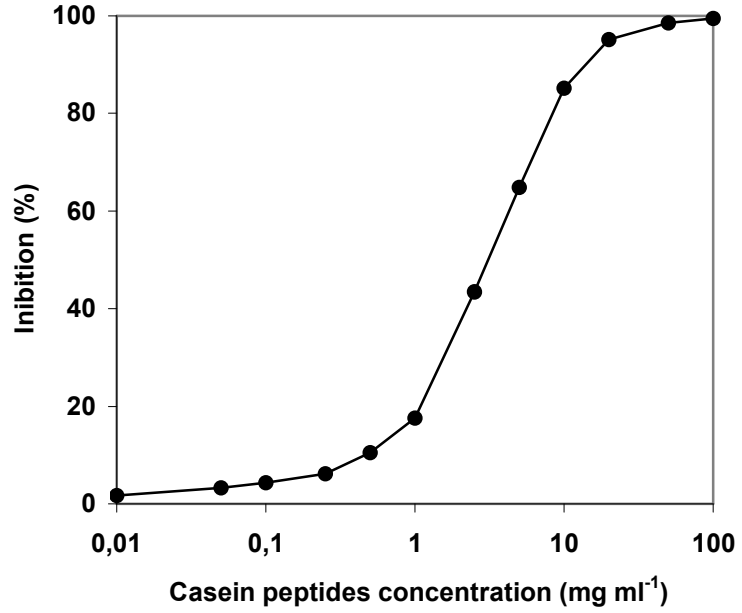

Fig.4. Rossini et al., 2007

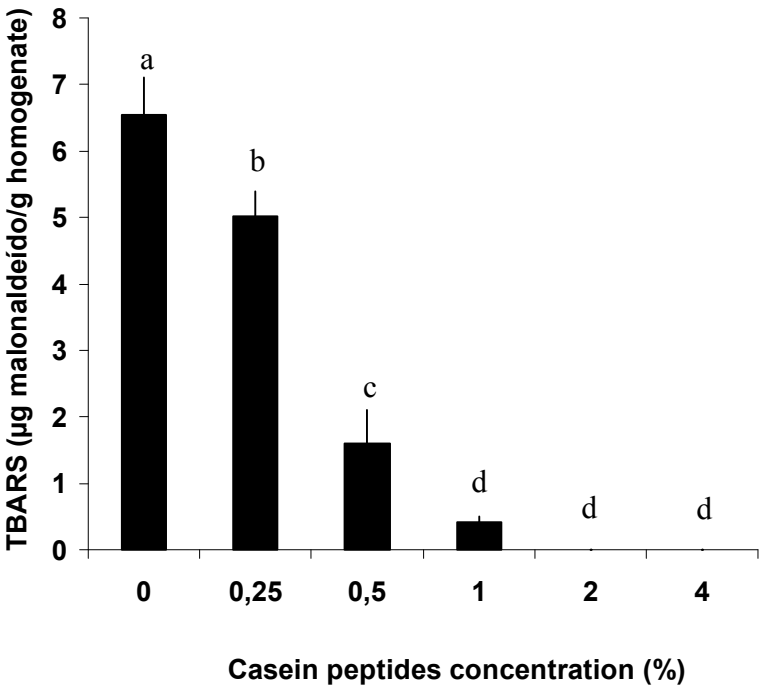

Fig.5. Rossini et al., 2007

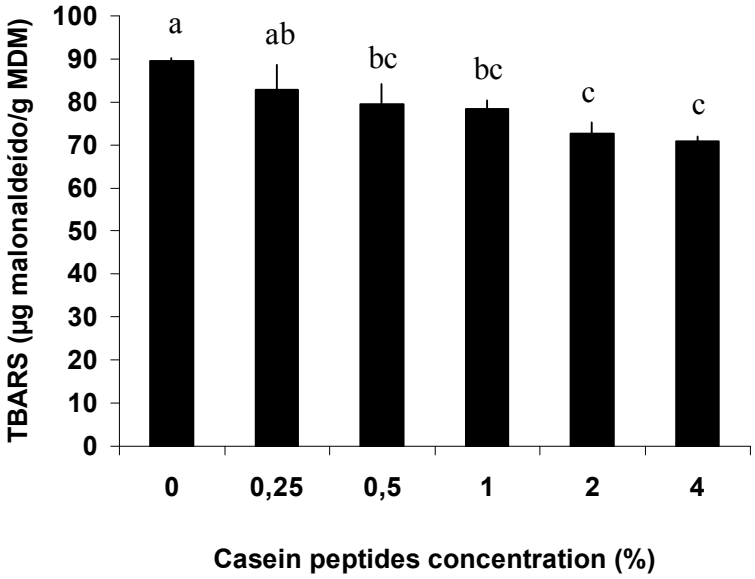

Fig. 6. Rossini et al., 2007

2.2 Changes in white chocolate during storage - Non-enzymatic browning reactions.

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Abstract

The main problem that limit the white chocolate's shelf-life is browning. Non-enzymatic browning reaction and lipid oxidation were involved directly in the browning of white chocolate. Thus, parameters that indicated the fat alteration and non-enzymatic reactions were evaluated in three different samples of white chocolate manufactured in laboratory. One sample with 0.2% (on cocoa butter) of the synthetic antioxidant Grindox 562, other with 0.2% (on cocoa butter) of the natural antioxidant casein peptides and the third sample without any kind of the antioxidant. Samples were stored at 20 ± 2ºC and 28 ± 2ºC. The results of analysis indicated that samples stored at 20ºC showed significant better results than those stored at 28ºC, regarding the conservation of white chocolate. Also, the addition of any antioxidant did not influenced significantly the results, indicating that the main parameter governing the alterations of white chocolate during its shelf life is the storage temperature.

Keywords: Casein peptides; White chocolate; Lipid oxidation; Maillard reaction.

1. Introduction

Appearance involves all visual phenomena characterizing objects, including gloss, color, shape, roughness, surface texture, shininess, haze and translucency (Briones et al., 2006). In confectionery products manufactured such as white chocolate the development of brown colors is one of their main problems that limit the shelf-life. The brown color is more relevant in white chocolates that are often sold in transparent packaging because the consumer can compare them with a white product. Naturally, consumers expect that white chocolate should have a white to pale yellow color and the presence of a dark yellow color, or even light brown color, is undesirable (Vercet, 2003). The ingredients of white chocolate react with each other during processing and storage resulting in the development of browning color.

The ingredients of the confectionery white chocolate are sugar, milk solids (mainly whole milk powder; but in some formulations skimmed milk powder and whey powder could be used), cocoa butter, soy lecithin and vanillin. Milk solids have undoubtedly been responsible for the great increase in the consumption of white chocolate (Muresan et al., 2000). These solids have commonly been used in the enhancement of nutritional and technological qualities of another wide range of different foods as well as in adding the desired flavour to them (Tunçtürk and Zorba, 2006). However, it occur chemical interaction between the milk proteins and the aldehyde groups of reducing sugar (lactose). It is called of Maillard reaction, non-enzymatic browning reactions, and the development of flavor and color are related to temperature, time and presence of water (Muresan et al., 2000). Maillard reaction that occur between reducing sugars and proteins, peptides or amino acids during the processing and/or storage of foods (Moreno et al., 2006). Due high percentage of fat, from cocoa butter and milk, the lipid oxidation could be involved in color evolution of white chocolate.

Lipid oxidation is a serious problem because it not only produces off-flavors but also decreases the nutritional quality, safety and shelf-life of the food (Shih and Daigle, 2003). Therefore, the control of lipid oxidation in food products is desirable, and the benefits of antioxidants in food storage have been studied by many researchers (Sakanaka et al., 2005). Synthetic antioxidants, such as butylated hydroxylanisole (BHA), butylated hydroxytoluene (BHT),

propyl gallate (PG), and tert-butylhydroquinone (TBHQ), have been commonly used to suppress the formation of free radicals, preventing lipids from oxidation and food spoilage. Although these synthetic reagents are efficient and relatively cheap, special attention has been given to natural antioxidants because of a worldwide trend to avoid or minimize the use of synthetic food additives (SHIH and DAIGLE, 2003).

The aim of this study was to investigate the effect of antioxidants in the color alteration of white chocolate during its shelf-life. Two different antioxidants, synthetic Grindox 562 and casein peptides resulting from hydrolysis with enzyme Flavourzyme, were tested during storage at two temperatures 20±2 and 28±2ºC. Parameters of non-enzymatic browning reactions and lipid oxidation were evaluated for the period of ten months.

2. Materials and methods

2.1 Reagents

The sample ofwhite chocolate was from Florestal Alimentos S/A (Porto Alegre, Brazil). Glucose and 2-thiobarbituric acid were obtained from Sigma. Chloroform, acetic acid, and trichloroacetic acid were from Nuclear (Brazil). Commercial antioxidant Grindox 562 was from Danisco (Copenhagen, Denmark), casein was from Farmaquimica (São Paulo, Brazil). Flavourzyme™ (endoprotease and exopeptidase from Aspergillus oryzae) was from Novozymes Latin America (Bento Gonçalves, Brazil). All other reagents were of analytical grade.

2.2 Manufacture of products

Three different formulations of white chocolate samples were utilized: a white chocolate elaborated with a commercial antioxidant Grindox (0.2%, on the cocoa butter), a white chocolate elaborated with a natural antioxidant from hydrolysis of casein (0.2%, on cocoa butter) and a white chocolate elaborated without antioxidant. Formulations of products are given in Table 1. The chocolate production procedure follows the traditional process, consisting of mixing the

ingredients (powder milk, soy lecithin, cocoa butter, sugar and/or antioxidant), refining and conching (Beckett, 1988). Then the white chocolate was tempered and molded in tablets. The tablets had a weight of 25g and were packed with a polypropylene plastic film. Samples were stored in the two temperatures: 20ºC ± 2ºC and 28ºC ± 2ºC and at a relative humidity of 65%.

2.3 Samples preparation

The technique described for Vercet (2003) for preparation of the samples for analyses was used. For each determination, all the analyses were performed in triplicate. Then, several batches of 4 g were defatted with 25 mL of a mixture of chloroform/methanol (95:5) and centrifuged at 3000 g during 30 min. The solvent fraction was decanted and solvent was evaporated. The fat obtained was analysed for the peroxide value, acidity index and UV absorbance.

The defatted pellet was suspended in water at 50 ºC in a 50-mL volumetric flask and vigorously stirred. Then 0.5 mL of Carrez I reagent was added and afterwards 0.5 mL of Carrez II reagent. The solution was left to rest for 10 min and the volume was adjusted to 50 mL with distilled water. The solution was filtered and the filtrate was used for the analysis of reducing sugars.

2.4 Peroxide value

The peroxide value was performed by the AOAC method (AOAC; 1990). Extracted fat (5 g) were placed in a 100-mL flask and dissolved in 30 mL of an acetic acid-chloroform solution (3:1). Then, 0.5 mL of a saturated solution of KI was added. It was left to stand in the dark for 2 min with a gentle stirring and then 30 mL of water were added. The liberated iodine was titrated with 0.01 N Na2S2O3. When the brown color tended to disappear, 1 mL of a 1% soluble starch solution was added to give a better control of the end point.

2.5 Acidity index

Acidity index was determined by titration. Extracted fat (1 g) was dissolved in 50 mL of diethyl ether and titrated with KOH (0.1 N) dissolved in methanol. Three drops of an indicator were added to give a better control of the end point.

2.6 Color determination

The surface color of the chocolate tablets was also measured using a CM Colorimeter model 500-d Series (Minolta, Japan). The parameters determined were luminosity or brightness (L* = 0 black and L* = 100 white), red-green component (-a* = greenness and +a* = redness) and yellow-blue component (-b* = blueness and +b* = yellowness); where L*, a* and b* values at the considered storage time were considered with respect to those obtained in just manufactured samples (time zero).

2.7 Water activity

Water activity (at 25 ºC) was determined measuring directly in a water activity instrument (Aqualab 3TE-Decagon, Pullman, USA).

2.8 Reducing sugars

According to Miller (1959), 100 µL of an aliquot of samples were reacted with 1000 µL of 3,5-dinitrosalycílic acid (DNS) reagent. The sample was then heated in a boiling water bath for 5 min and cooled to room temperature. The developed color was measured at 550 nm using a Hitachi U1100 Spectrophotometer. A standard curve was developed using glucose (0-5mg/mL).

2.9 Thiobarbituric acid reactive substances (TBARS)

The thiobarbituric acid reactive substances (TBARS) method was performed by standard procedure (IAL; 1985). White chocolate samples (5 g) were placed in a 50-mL flask, dissolved in chloroform and transferred to 100-mL volumetric flask and the volume was completed with chloroform. Then, 5 mL solution was transferred to a centrifuge tube of 20 mL, to which 5 mL of trichloroacetic acid (TCA) 10% was added before centrifugation at 3000 x g for 10 min. After centrifuged, 4 mL of supernatant was mixed with 1.25 mL of thiobarbituric acid. The sample was then heated in a boiling water bath for 10 min and cooled to room temperature. The absorbance was measured at 530 nm using spectrophotometer Hitachi U-1100. Thiobarbituric acid reactive substances were calculated from a standard curve of malonaldehyde (MDA), a breakdown product of tetraethoxypropane (TEP).

2.10 Statistical analysis

Results were expressed as means ± S.D. of three independent determinations. Data were evaluated statistically by one-way analysis of variance (ANOVA) and Tukey test.

3. Results

The evolution of parameters referred to non-enzymatic browning reaction was monitored for ten months. The data in Figure 1 show a curve obtained under experimental conditions, resulting in an increase in peroxide value as a function of time with all compositions and temperature. In the end of ten months of shelf life, the sample without antioxidant and submitted to the temperature of 28ºC had the highest increase of the peroxide value, significantly different from the other samples. Comparing to the samples with antioxidants to 28ºC, these did not show any difference among themselves, but showed difference from the samples without antioxidant at the same temperature and also from all samples submitted to the temperature of 20ºC. The

samples stored at 20ºC, did not present significant difference and showed the least formations of peroxides.

In Figure 2, it is observed that the water activity value of the samples increased during the ten months of shelf life. There was no significant difference only of the referred values in the samples submitted to the same temperature. Among the samples, the ones submitted to 20ºC had less increase in the value.

In relation to the color, Figure 3A, it is possible to observe the increase of the a* parameter, evidencing the rise of intensity of the red color. At the end of the storage period, it was verified significant differences among the samples stored at different temperatures, however, there was no difference when they were compared to samples submitted to the same temperature.

In the color expressed by the b* parameter, after ten months of shelf life, there was a rise of intensity of the yellow color for all samples as shown in Figure 3B. The samples without any kind of antioxidants, both for the temperature of 20ºC and 28ºC, showed no difference among themselves and had the most accentuated development of the yellow color, but showed significant difference when compared to the other samples, which independently from the temperature, showed no statistic difference among themselves.

On the other hand, in the L* parameter – Figure 3C – it was observed, after ten months, a decrease of brightness, however, there was no significant difference among all samples.

The results of the analysis realized for the determination of the acidity index and thiobarbituric acid reactive substances showed a significant increase in its values throughout time – Figure 4 and 5 – but no significant difference among all samples at the end of the storage period.

For the concentration of reducing sugars, the results showed the diminution of this concentration throughout time - Figure 6. In relation to the concentration, at the end of the storage period, it was verified significant differences among the samples stored at different temperatures, however, there was no difference observed when they were compared to samples stored at the

same temperature. The samples submitted to the temperature of 28ºC presented the highest losses in the quantity of these sugars.

4. Discussion

In this work, samples of white chocolate were produced with and without the addition of antioxidants and stored at two different temperatures, 20± 2ºC e 28 ± 2ºC. The percentage of solid fat in a cocoa butter at different temperatures is shown in Table 2 (Subramaniam, 2000), indicating that the ideal temperature for the storage of white chocolate is around 20ºC.

The utilization of natural antioxidant derived from the enzymatic hydrolyses of casein is justified by information from Kitts (2005) which says that these peptides derived from the casein (CPP) have an affinity to quenching Fe^{2+}. Associated with this activity, the CPP also effectively suppressed Fenton reaction-induced site-specific and non site-specific deoxyribose oxidation. In addition, these peptides were effective at reducing 2,2`-azobis(2amidinopropane) dihydrochloride; (AAPH-) and Fe2+ - induced liposomal peroxidation and showed direct scavenging affinity for the hydrophilic 2,2`-azinobis-3- ethylbenzothiazoline-6-sulfonic acid; (ABTS) radical. It can be concluded that peptides derived from bovine casein have both primary and secondary antioxidant properties that specifically involve direct free radical scavenging and sequestering of potential metal prooxidants.

The Figure 1 shows the value related to the peroxide value. The highest value was obtained from the sample stored at the temperature of 28ºC and without antioxidant, with value around 7.0 meq O2/kg fat, after the 10 months of storage. In this same analysis, Vercet (2003) obtained values of 9.5 meq O2/kg fat, after 15 months of storage at 20ºC. Mattisek et al. (1998) states that, although these values have raised throughout time, they are still lower than 10 meq O2/kg fat indicating that the alterations related to the lipid degradation are still in the initial stage.

Through the results of the analysis related to the acidity and thiobarbituric acid reactive substances, it was possible to reinforce the result described above. Since the results show no significant difference among the samples with and without the addition of antioxidants and also

did not depend on the temperature to which they were exposed, evidencing this way, that the lipid degradation, although it is related to one of the main deteriorative problems related to products with high quantity of lipids, being one of the first mechanisms of quality loss of food products (Haak et al., 2006), did not influence significantly the color alteration of the white chocolate samples in the conditions they were analyzed. Also, Vercet (2003), in his analyses, detected that there is no important variation in the composition of fats during period of 15 months. Fatty acid methyl esters profiles for fats extracted from chocolates at the beginning and at the end of the experiments are the same. This indicates that there has not been much loss in the unsaturated fatty acids present in the chocolate.

As shown in Figure 2, water activity increased significantly in all samples throughout time. Water activity of chocolate is normally between 0.4 and 0.5, and depends on several factors: the raw materials used, the surface area of the materials, the amount of lecithin and the processing conditions (temperature and moisture of refining and conching). It concludes that even at the moderate water activity of chocolate, it is very difficult for it to pick up moisture because the fatty surface will protect it from the incoming water (Richardson, 1987). Vercet (2003) considers two ingredients: amorphous sugars and lecithin. Amorphous sugar is a metastable form and tends to crystallize under the influence of a number of factors, mainly temperature and moisture (Gloria et al., 2001). There are two important sugars in white chocolate; sucrose and lactose. During the milling of sucrose to reduce particle size and incorporate it into the chocolate, high temperatures are reached and a considerable amount of amorphous sucrose is formed (Niediek, 1991). But lactose is different; there is a high percentage of lactose in the amorphous state and it is thermodynamically unstable and hygroscopic, absorbing moisture from the surrounding and subsequently plasticizing. When crystallization occurs, water is suddenly liberated and local water activities could increase. The temperature at which this transition from an amorphous solid-state to a viscous rubbery state occurs is known as the glass transition temperature. The glass transition temperature can fall below ambient temperature when the powder absorbs sufficient moisture during storage (Ibach & Kind, 2007). In the results shown, it was observed significant difference for the values of water activity among the samples stored at different temperatures,

with higher values for the samples stored at 28ºC, indicating that this influence in the quantity of free water in the samples, through the crystallization of lactose, according to what it was explained by the authors cited. Although the values of water activity have raised, the highest values verified for this parameter did not reach 0.6, prohibiting this way, the microbial growth, but according to Richter & Lannes. (2007), the lowest value of water activity observed for the bacteria growth was 0.75 and Aw of xerophilic fungus and osmophilic levedures of 0.65 and 0.61, respectively. Nevertheless, although this raise of water activity does not influence the microbial growth, it contributes for the development of the reaction of Maillard. Caboni et al. (2005), says that although the optimal range of water activity for Maillard reaction is 0.52 – 0.75, he observed a small increase in the development of this during the storage of egg powder with water activity of 0.32– 0.35. Moreno et al. (2006), observed the occurrence of this reaction for values of water activity of 0.44 and temperature of 30ºC in onions. Garcıa-Baños et al. (2005) visualized the development of Maillard reaction in powder enteral formulas during storage at 30ºC and water activity of 0.44. A decrease of total carbohydrates was observed after of the 6 months of storage.

The increase of the a* parameter throughout time, indicates that the white chocolate is becoming darker, possibly due to the Maillard reaction and this fact is verified by the diminution of the reducing sugars concentration, being this decrease higher to the temperature of 28ºC and higher water activity.

In white chocolate, the initial stage of the Maillard reaction involves interaction between the ε-NH2 of protein-bound lysine with lactose, dissacaryde formed by glucose and galactose found in the milk in concentrations of 4.5 a 5.0 g/100mL (Messia et al., 2007), to form lactuloselysine [ε -(deoxylactose) lysine] (Friedman, 1996).

The result of the b* parameter, which represents the intensity of the yellow color, demonstrated that the presence of antioxidants might have act to prevent from accentuated development of yellow color, since the samples without any kind of antioxidants presented the highest values in this analysis, independent from the temperature at which they were stored.

The L* parameter, that is the brightness in white chocolate, has diminished in all samples throughout time, with no significant difference among the samples at the final time, indicating that this parameter is not influenced neither by the addition of antioxidants nor by the temperature.

5. Conclusion

Among the samples analyzed, the ones that presented the best results, maintaining in a stable way its characteristics, were those stored at the temperature of 20 ± 2ºC, independent from the addition of antioxidants. Besides, as shown by Vercet (2003), the development of browning in white chocolate is due to the non-enzimatic browning reactions and the ambient conditions during the storage period. The lipid oxidation was evidenced, but in its initial stage, not being source of significant alterations among the samples.

Acknowledgements

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Table 1.

Composition of white chocolate used as sample

Ingredient	Percentage
Sugar	44.5
Cocoa butter	29.6
Whole milk powder	17.1
Skimmed milk powder	8.5
Soy lecithin	0.3

Table 2.

Solid fat content to different temperatures of cocoa butter (Subramaniam, 2000)

Temperature (°C)	Solid fat (%)
0	83
20	83
25	76
30	55
35	1
40	0

Figure Legends

Fig. 1. Evolution of peroxide value during storage: 20ºC white chocolate with synthetic antioxidant (●), 20ºC white chocolate with natural antioxidant (×), 20ºC white chocolate without antioxidant (■), 28ºC white chocolate with synthetic antioxidant (*), 28ºC white chocolate with natural antioxidant (▲), 28ºC white chocolate without antioxidant (♦).

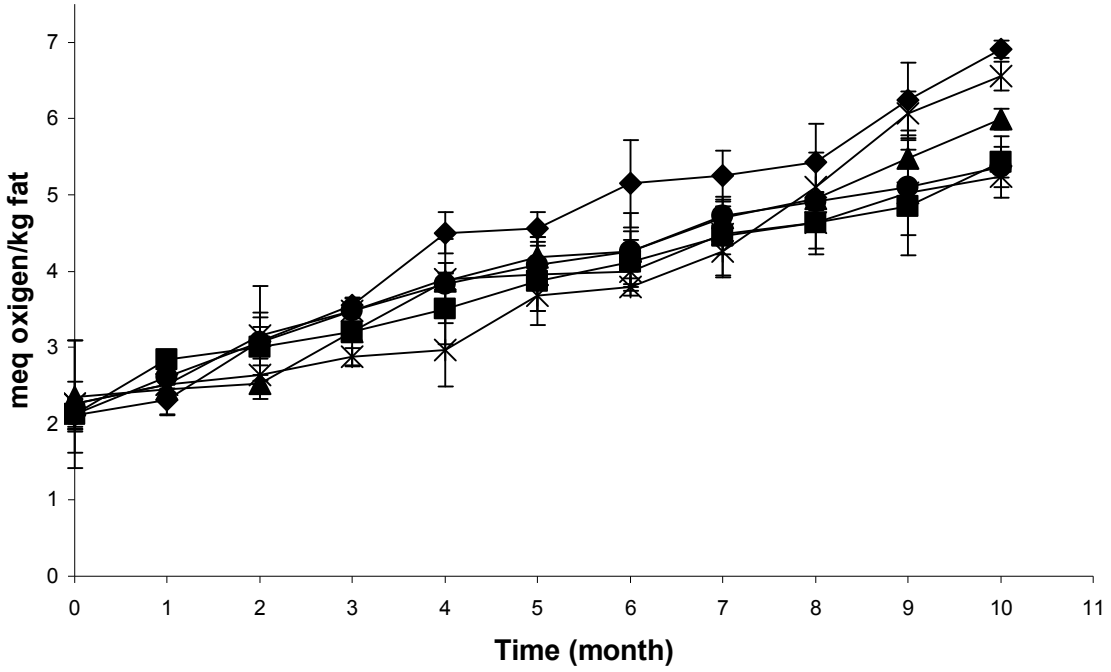
Fig. 2. Evolution of water activity during storage: 20ºC white chocolate with synthetic antioxidant (●), 20ºC white chocolate with natural antioxidant (×), 20ºC white chocolate without antioxidant (■), 28ºC white chocolate with synthetic antioxidant (*), 28ºC white chocolate with natural antioxidant (▲), 28ºC white chocolate without antioxidant (♦).

Fig. 3. Evolution of parameter Color a* (A), Color b* (B) and Color L* (C) during storage: 20ºC white chocolate with synthetic antioxidant (●), 20ºC white chocolate with natural antioxidant (×), 20ºC white chocolate without antioxidant (■), 28ºC white chocolate with synthetic antioxidant (*), 28ºC white chocolate with natural antioxidant (▲), 28ºC white chocolate without antioxidant (♦).

Fig. 4. Evolution of Acidity Index during storage: 20ºC white chocolate with synthetic antioxidant (●), 20ºC white chocolate with natural antioxidant (×), 20ºC white chocolate without antioxidant (■), 28ºC white chocolate with synthetic antioxidant (*), 28ºC white chocolate with natural antioxidant (▲), 28ºC white chocolate without antioxidant (♦).

Fig. 5. Evolution of Thiobarbituric acid reactive substances (TBARS) during storage: 20ºC white chocolate with synthetic antioxidant (●), 20ºC white chocolate with natural antioxidant (×), 20ºC white chocolate without antioxidant (■), 28ºC white chocolate with synthetic antioxidant (*), 28ºC white chocolate with natural antioxidant (▲), 28ºC white chocolate without antioxidant (♦).

Fig. 6. Evolution of Reducing Sugars during storage: 20ºC white chocolate with synthetic antioxidant (●), 20ºC white chocolate with natural antioxidant (×), 20ºC white chocolate without antioxidant (■), 28ºC white chocolate with synthetic antioxidant (*), 28ºC white chocolate with natural antioxidant (▲), 28ºC white chocolate without antioxidant (♦).



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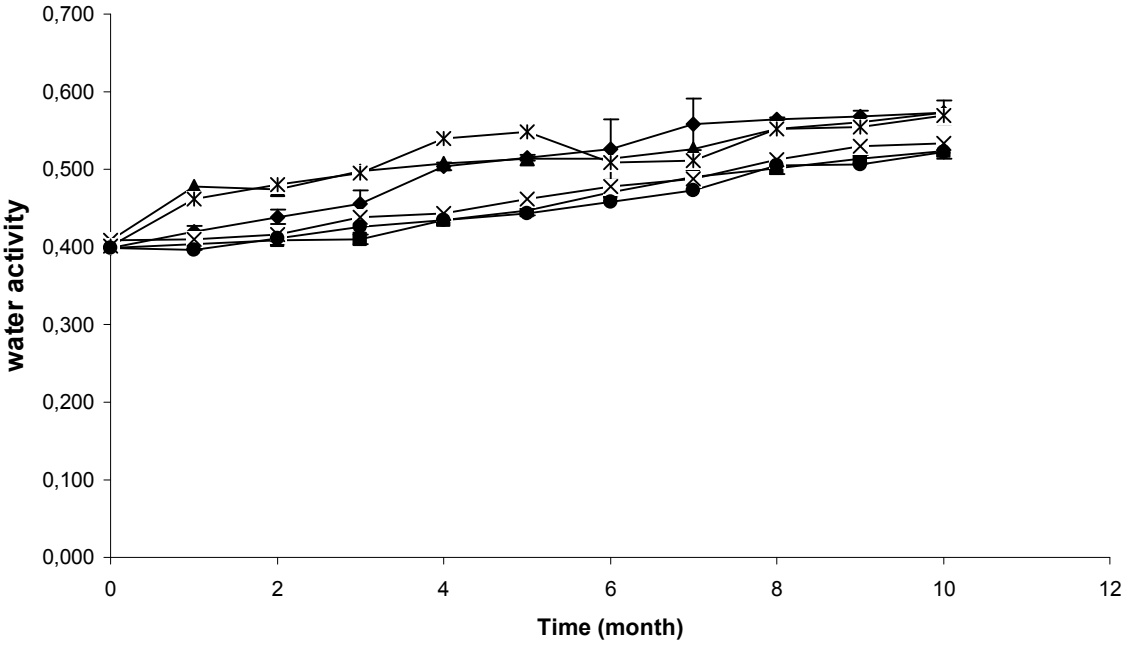

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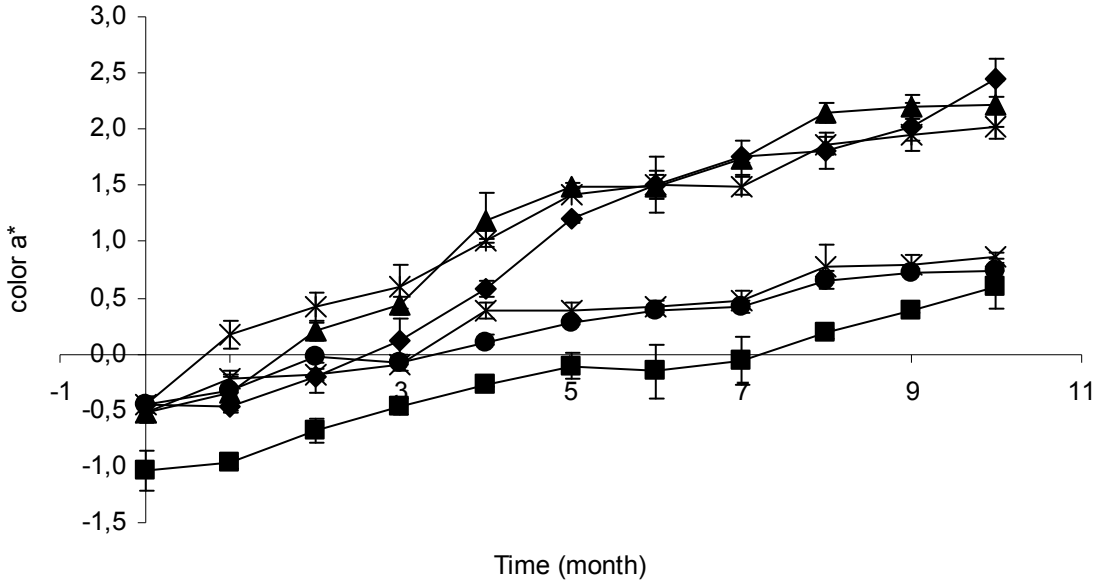
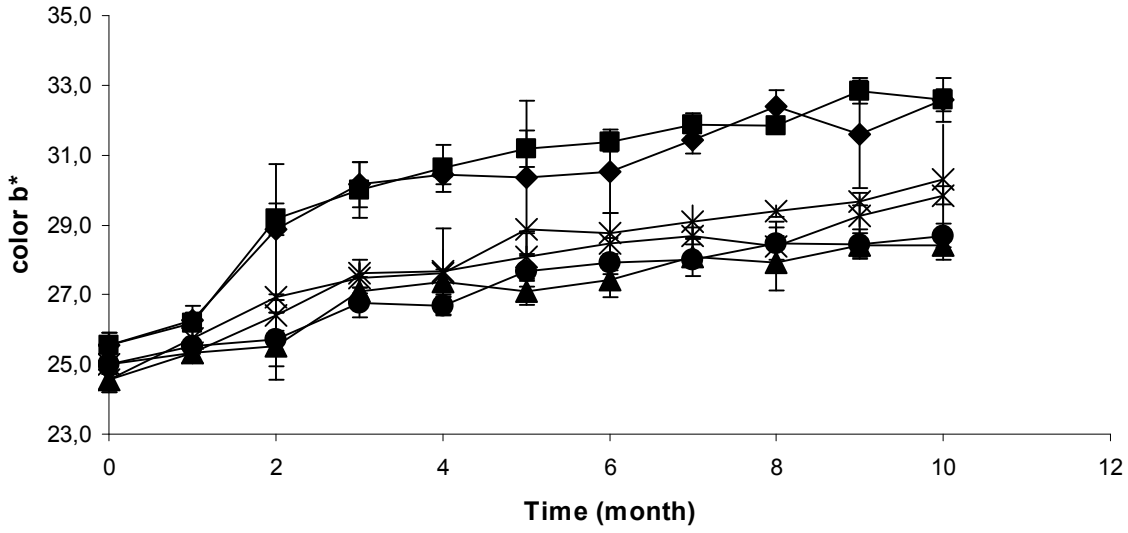


Fig. 3A. Rossini et al., 2007.



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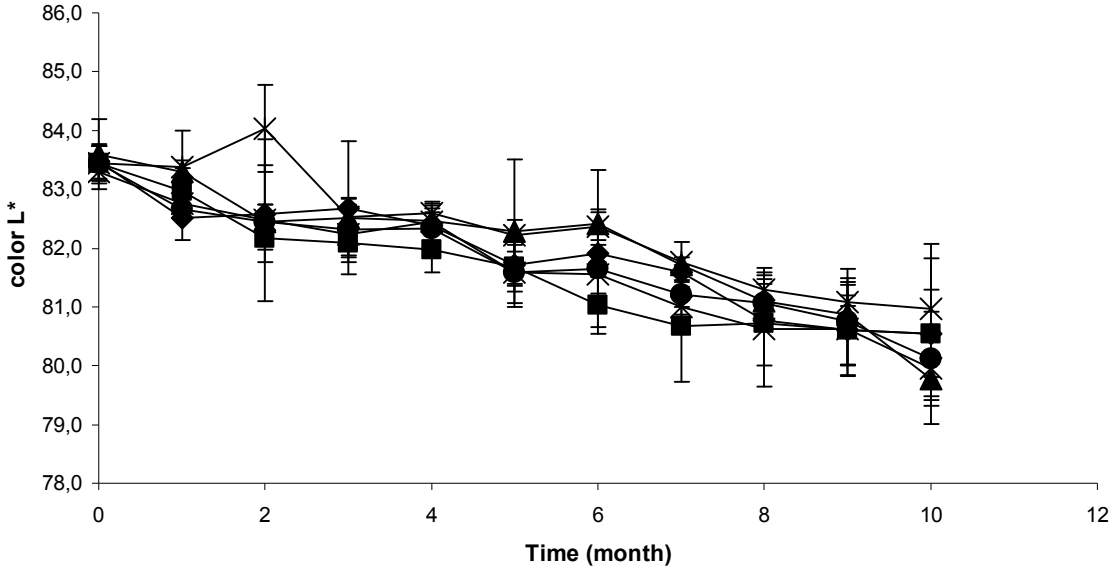

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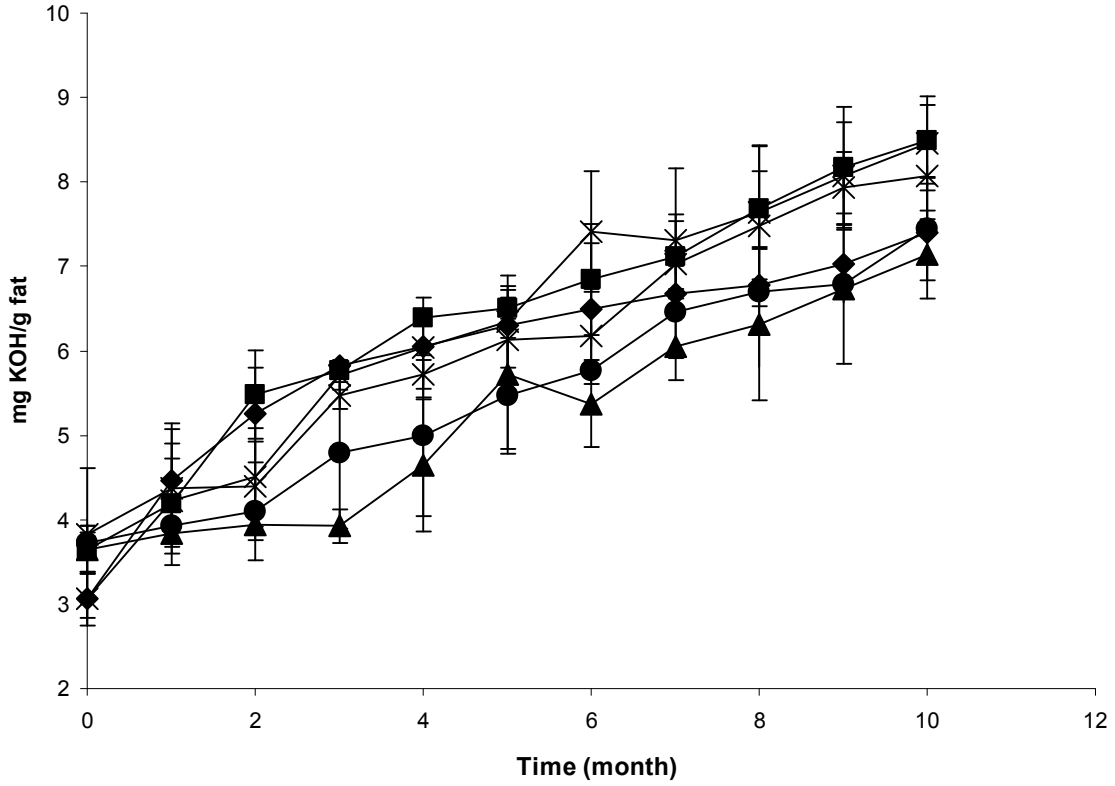

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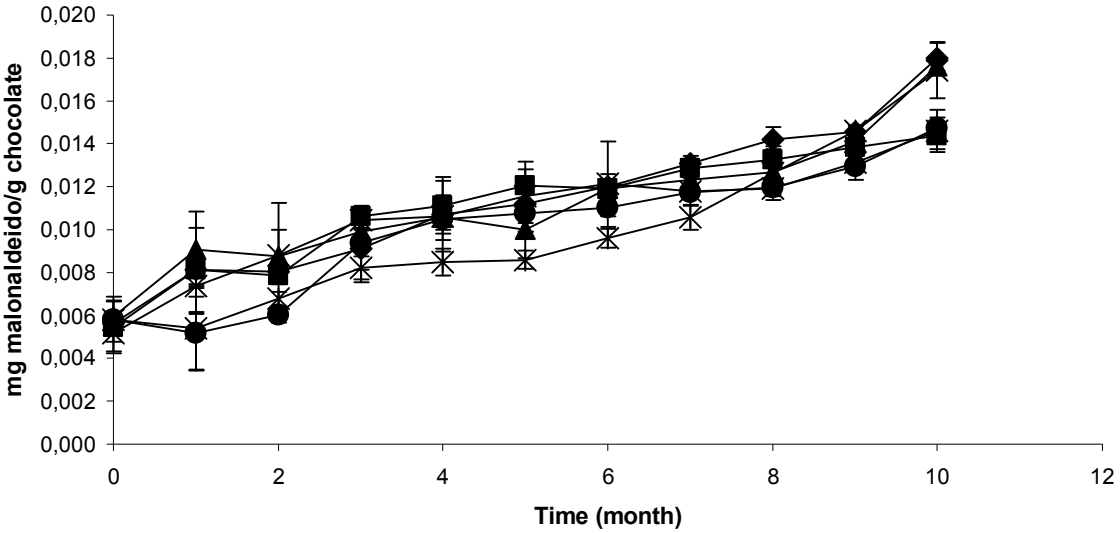

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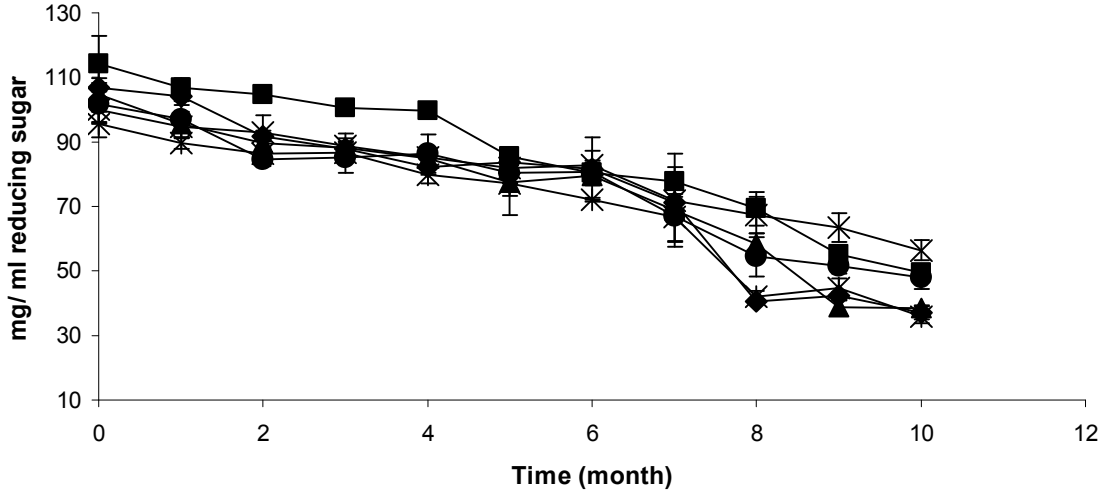

Fig. 6. Rossini et al., 2007

3 DISCUSSÃO GERAL

Na busca por produtos naturais, muitos pesquisadores têm estudado a ação antioxidante da caseína hidrolisada bem como sua utilização em alimentos. Com base nestas informações, o presente trabalho abordou o potencial antioxidante da hidrólise enzimática da caseína, in vitro, caracterizando-o e adicionando-o em amostras de carne moída, de carne mecanicamente separada de ave (CMS) e de chocolate branco.

A ação do antioxidante natural, tanto primária quanto secundária, através do seqüestro de radicais livres e metais pro-oxidantes, derivada da hidrólise enzimática da caseína, justifica-se pelas informações de Kitts (2005), o qual relata a afinidade dos peptídeos oriundos da referida hidrólise no seqüestro do Fe2+ e do 2,2`-azinobis-3-ethylbenzothiazoline-6-ácido sulfónico; (radical ABTS), na inibição da reação de Fenton, na redução do 2,2`-azobis (2amidinopropane) dicloridrato; (AAPH-) e do Fe2+ - induzida pela peroxidação.

Segundo Lahl e Braun (1994), a proteólise tem sido utilizada para modificar as propriedades funcionais das proteínas, tais como solubilidade, gelificação, e emulsificação, e para o desenvolvimento de formulações hipoalergênicas. Além disso, conforme Clare e Swaisgood (2000), vários estudos têm reportado à importância de peptídeos derivados da caseína em funções biológicas. Por sua vez, Sinha et al. (2007) afirmam que peptídeos bioativos em proteínas do leite são latentes até serem ativados pela proteólise enzimática, também, que peptídeos do soro de leite obtidos por hidrólise com proteases de fungos mostraram atividade anti-hipertensiva e, ainda, que peptídeos de caseína derivados de queijo mostraram atividade antimicrobiana e antioxidante.

Inicialmente, neste trabalho estudou-se a obtenção do antioxidante natural mediante a hidrólise enzimática da caseína. No desenvolvimento deste hidrolisado testou-se duas enzimas proteolíticas comerciais, a Alcalase e a Flavourzyme, uma vez que estas enzimas têm sido descritas como eficientes na hidrólise protéica, como mostram os trabalhos de Zhu et al. (2006) que utilizou a Alcalase para hidrolisar gérmen de trigo e Peña-Ramos et al. (2002) que usou tanto a Alcalase quanto a Flavourzyme para hidrolisar proteína de soja.

Conforme os dados apresentados no primeiro artigo “Production and characterization of casein peptides with antioxidants properties and their effects on lipid oxidation in beef homogenates and MDM”, a caseína foi dissolvida em água destilada, pH 8.0, a uma concentração de 13%, sendo hidrolisada pelas proteases (0.4/100

enzima/substrato) durante 4 horas, sob temperatura de 50ºC e agitação constante. Após a obtenção destes hidrolisados por ambas as enzimas, realizou-se a caracterização dos mesmos.

No que tange à referida caracterização dos hidrolisados, esta foi composta das análises de proteína solúvel, aminoácidos livres, cromatográfica de gel filtração, eletroforese em gel de poliagrilamida e atividade antioxidante com o radical ABTS.

Observou-se que, a hidrólise enzimática com a Flavourzyme apresentou melhores resultados tanto no conteúdo de proteína solúvel quanto no de aminoácidos livres, como demonstrados nas Figuras 1A e 1B - Artigo 1. Convergindo com estes resultados, as análises de cromatografia de gel filtração e eletroforese em gel de poliacrilamida indicam que a hidrólise da caseína com a Flavourzyme obteve peptídeos de tamanhos menores aos da Alcalase, vide Figura 2 e 3 – Artigo 1. Autores como Sakanaka et al. (2005) sugerem que o tamanho dos peptídeos deva ser um dos principais fatores a conferir propriedades antioxidantes a estes hidrolisados.

Através da análise da atividade antioxidante in vitro, com o radical ABTS, constatou-se a superioridade do hidrolisado com a enzima Flavourzyme, quando este apresentou valores de 23.9 ± 2.3 mg Eq vitamina C mL-1 comparados com 7.7 ± 0.8 mg Eq vitamina C mL-1da Alcalase.

Com base nestas análises, cujos resultados apresentados com a enzima Flavourzyme mostraram-se significativamente superiores aos da Alcalase, a continuidade dos experimentos foi realizada somente com o hidrolisado desta enzima.

A caracterização do perfil de aminoácidos do hidrolisado com a enzima Flavourzyme, foi comparado ao perfil da caseína intacta, sendo este perfil de aminoácido distinto entre as duas amostras, indicando que a hidrólise da caseína não causa perda de aminoácidos, conservando o seu elevado valor nutritivo. Além disso, o hidrolisado apresentou cisteína livre – aminoácido conhecido pela sua atividade antioxidante (Kitts, 2005).

Ao ser avaliada a afinidade de quelar metais, pelos peptídeos da caseína, pelo método do radical ABTS, in vitro, obteve-se atividade máxima de 99.52% a uma concentração de 100 mg mL-1 (Figura 4 - Artigo 1). Kitts (2005) encontrou atividade máxima de 91.8% a 1.0 mg mL-1 de peptídeos de caseína cálcio (CCPs). Dessa forma, este autor, sugere a aplicação destes peptídeos em carne, com o intuito de ajudar na prevenção da oxidação lipídica e formação de flavor desagradável, aumentando sua

vida útil. Por sua vez, Lee et al. (1997) refere que produtos cárneos são eficazes meios para investigar o efeito de antioxidantes contra a peroxidação lipídica.

Assim, estudou-se a atividade antioxidante dos referidos peptídeos adicionando-os à carne moída e à carne mecanicamente separada de ave (CMS). Quando incorporados (2.0%) à carne moída, observou-se 100% de inibição da oxidação lipídica, porém, somente 21% em CMS. Sakanata et al. (2005) obteve 69.7% de inibição ao adicionar 2.0% de peptídeos de cálcio caseína (preparados pela hidrólise enzimática microbiológica de cálcio caseína) à carne moída e Lee & Hendricks (1997) observou 76.2% de inibição 20 mM de carnosina (um dipeptídeo endógeno encontrado no esqueleto da maioria do vertebrados).

Após obter-se resultados satisfatórios na adição do hidrolisado de caseína em carnes, este foi adicionado a amostras de chocolate branco, comparando-se a um antioxidante comercial, o Grindox 562.

De acordo com os resultados reportados no segundo artigo “Changes in the color of white chocolate during storage - Non-enzymatic browning reactions”, as amostras de chocolate branco foram produzidas com e sem adição de antioxidantes, armazenadas em duas diferentes temperaturas, 20 ± 2ºC e 28 ± 2°C, e durante 10 meses. O percentual dos antioxidantes acrescidos nas amostras de chocolate branco foi o mesmo, independente do antioxidante utilizado, e condizente com as informações do fabricante do antioxidante comercial, sugerindo este a quantidade de 0.2% do percentual de manteiga de cacau existente no chocolate.

A porcentagem de gordura sólida na manteiga de cacau a diferentes temperaturas é mostrada na Tabela 2 – Artigo 2 (Subramaniam, 2000), indicando que a temperatura ideal para armazenamento do chocolate branco encontra-se na faixa de 20 ºC, já que nesta temperatura há 83% da manteiga de cacau solidificada. A temperatura de 28°C foi também utilizada à verificação da influência da temperatura na vida útil do chocolate branco. A fim de verificar as alterações relativas à cor e à oxidação lipídica, diferentes análises foram realizadas com periodicidade mensal.

A Figura 1 – Artigo 2 – demonstra os valores relativos ao índice de peróxidos. O valor máximo foi obtido com a amostra armazenada a 28 ºC e sem antioxidante, sendo próximo a 7,0 meq O2/kg gordura, após os 10 meses de armazenamento. Nesta mesma análise, Vercet (2003) obteve valores de 9,5 meq O2/kg gordura, após 15 meses de armazenamento, a 20 ºC. Mattisek et al. (1998) afirma que, embora estes valores

tenham aumentado ao longo do tempo ainda encontram-se abaixo de 10 meq O2/kg gordura, indicando as alterações relativas à degradação lipídica ainda em estágio inicial.

Através dos resultados das análises relativas à acidez e substâncias reativas ao ácido tiobarbitúrico, pôde-se ratificar o resultado acima descrito. Os resultados das referidas análises não apresentaram diferença significativa entre as amostras, com ou sem adição de antioxidante, independentemente da temperatura a que foram expostas. Evidencia-se, pois, que a degradação lipídica, embora seja identificada como um dos principais problemas deteriorativos relacionados a produtos de alto teor lipídico e sendo um dos primeiros mecanismos de perda de qualidade de produtos alimentícios (Haak et al., 2006), não influenciou significativamente na alteração da cor das amostras de chocolate branco nas condições que foram analisadas. Além disso, Vercet (2003) demonstrou em suas análises não haver variação considerável na composição do perfil de metil éster de ácidos graxos extraídos da gordura do chocolate branco, durante o período de 15 meses. Isto indica a não ocorrência de perda significativa dos ácidos graxos insaturados presentes no chocolate.

Analisando-se a atividade de água (aw), com o tempo, esta demonstrou um aumento significativo em todas as amostras. Em chocolate tal parâmetro situa-se, normalmente, entre 0.4 e 0.5, e depende de vários fatores como: matérias-primas, superfície do material, lecitina e condições de processo (temperatura e umidade de refino e conchagem) (Richardson, 1987). Vercet (2003) sugere dois ingredientes a serem considerados para justificar o aumento na aw: a presença de açúcares amorfos e lecitina. Salienta-se que o açúcar amorfo é uma forma metaestável e tende a cristalizar-se sob a influência, principalmente, da temperatura e umidade (Gloria et al., 2001). No chocolate branco há dois importantes açúcares: sacarose e lactose. Durante a moagem da sacarose para reduzir a dimensão das partículas e incorporá-la ao chocolate, altas temperaturas são atingidas e uma quantidade considerável de sacarose amorfa é formada (Niediek, 1991). A lactose é diferente, há uma elevada porcentagem de lactose no estado amorfo, termodinamicamente instável e higroscópica, absorvendo umidade do ambiente e posteriormente plastificando-se. Quando ocorre cristalização há liberação de água e conseqüente aumento da aw. A temperatura de transição do estado sólido-amorfo para um viscoso é conhecida como temperatura de transição vítrea. A temperatura de transição vítrea pode ficar abaixo da temperatura ambiente quando os açúcares absorvem umidade durante o armazenamento (Ibach et al., 2007).

Nos resultados apresentados, verificou-se diferença significativa nos valores de aw entre as amostras armazenadas nas diferentes temperaturas, com valores superiores nas armazenadas a 28 ºC, indicando que esta influenciou no teor de água disponível nas amostras, através da cristalização da lactose, conforme referência dos autores acima mencionados.

Embora os valores de aw tenham se elevado, os máximos encontrados para este parâmetro não atingiram 0.6, não permitindo, assim, o crescimento microbiano, pois conforme Richter et al. (2007), o menor valor de aw relatado para o crescimento de bactérias foi de 0.75 e de mofos xerofílicos e leveduras osmofílicas de 0.65 e 0.61, respectivamente. No entanto, embora esse aumento na aw não influencie no crescimento microbiano, contribuem com o desenvolvimento da reação de Maillard. Caboni et al. (2005) informa que, embora o melhor intervalo de aw para o desenvolvimento da reação Maillard esteja entre 0.52 –0.75, detectou-se a presença desta durante o armazenamento de ovo em pó com aw de 0.32 – 0.35. Moreno et al. (2006) também observaram a ocorrência desta reação em valores de aw de 0.44 e temperatura de 30 ºC, em cebolas. Garcıa-Baños et al. (2005) visualizou o desenvolvimento desta mesma reação em formulações de pó enteral, durante o armazenamento a 30 ºC e 0.44 de aw, onde a diminuição total de hidratos de carbono foi observada no final de seis meses de armazenagem.

O aumento do parâmetro a* com o tempo, indica que o chocolate branco está tornando-se de coloração escura, possivelmente, em virtude da reação de Maillard, fato que é verificado pela diminuição da concentração de açúcares redutores. O maior decréscimo destes açúcares ocorreu à temperatura de 28 ºC, onde também foram observados os maiores valores de aw.

No chocolate branco, a fase inicial da reação Maillard envolve a interação entre ε-NH2 de proteínas vinculadas à lisina com a lactose, um dissacarídeo formado por glicose e galactose, encontrado no leite em concentrações de 4,5 um 5,0 g/100mL (Messia et al., 2007), formando a lactuloselisina [ε - (deoxilactose) lisina] (Friedman, 1996).

O resultado do parâmetro b*, ou intensidade de cor amarela, demonstrou que a presença de antioxidantes pode ter agido de uma forma a prevenir o desenvolvimento acentuado da cor amarela, visto que as amostras isentas de quaisquer antioxidantes apresentaram os maiores valores nesta análise, independente da temperatura na qual estiveram armazenadas.

De forma geral, entre as amostras analisadas, as que apresentaram melhores resultados, mantendo de forma mais estável suas características, foram aquelas armazenadas à temperatura de 20 ± 2ºC, independente da adição ou não de antioxidantes. Além disso, assim como mostrou Vercet (2003), o desenvolvimento da cor escura no chocolate branco é resultante de reações de escurecimento não-enzimático e de condições ambientais durante o armazenamento. Quanto à oxidação lipídica, esta também foi evidenciada, porém, não causou alterações significativas entre as amostras.

Diante do exposto, embora os resultados não tenham indicado influência significava na prevenção da diminuição de atributos de qualidade no chocolate branco, verifica-se que os mesmos indicam a eficiência da utilização do hidrolisado de caseína na ação antioxidante de produtos cárneos. Nesse contexto, sugere-se que o hidrolisado de caseína constitui-se numa boa fonte de antioxidantes naturais, apresentando-se como opção à crescente demanda por alimentos desta origem.

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