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Thalles Canton Trevisol

Papain as cosmetic exfoliant: *in vitro* effect in proteins of the skin and immobilization in beauty face masks of starch and carboxymethyl cellulose

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Certificamos que esta é a versão original e final do trabalho de conclusão que foi julgado adequado para obtenção do título de Doutor em Engenharia Química.

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Orientador

Florianópolis, 2023.

I dedicate this thesis to my parents and to all people that helped me during this journey. Especially, I dedicate this thesis to my godmother, Bernardett Trevisol (*in memory*), which always encouraged and praised my studies. This doctorate is, also, yours.

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“Make it stop

Let this end

All these years pushed to the ledge

But proud I stand, of who I am

I plan to go on living”

(Rise Against)

RESUMO EXPANDIDO

Introdução

A esfoliação é uma técnica que é utilizada para auxiliar a renovação celular da pele. Além disso, ela é conhecida por eliminar impurezas e auxiliar a penetração de formulações cosméticas. Os esfoliantes podem ser classificados com químicos, físicos ou enzimáticos, dependendo do ingrediente utilizado na sua produção. O setor de cosméticos está sempre em busca de inovação e alternativas para produzir novos produtos. O uso de enzimas tem se destacado nesse setor devido à sua especificidade de atuação, produção biológica e ser ecologicamente correto, diferente dos outros tipos de esfoliantes (GONÇALVES, 2021; PACKIANATHAN; KANDASAMY, 2011).

A esfoliação enzimática se baseia em imitar o processo de descamação da pele, que tende a diminuir com os anos, o que diminui a renovação do órgão e acarreta em aparências indesejadas (EGELRUD, 2000; PACKIANATHAN; KANDASAMY, 2011). Diferentes enzimas podem ser utilizadas para esse fim, destacando-se as enzimas proteolíticas, as quais tem capacidade de promover reações de hidrólise de proteínas. Papaína, bromelina, queratinases, colagenases e proteases microbianas são exemplos de enzimas que podem ser utilizadas para esse fim. Poucos estudos científicos avaliaram o uso de enzimas em cosméticos, apesar da existência de produtos no mercado com esse tipo de biocatalisador (SIM et al., 2000; SUNAR; KUMAR; DESHMUKH, 2016).

A papaína é a protease de origem vegetal mais comercial e industrialmente utilizada. Ela é extraída do látex do mamão (*Carica papaya*) e tem como destaque ampla operacionalidade e especificidade por substrato (FERNÁNDEZ-LUCAS; CASTAÑEDA; HORMIGO, 2017). Desse modo, essa enzima já vem sendo estudada e utilizada como agente de desbridamento de feridas (LANGER et al., 2013; PORSANI et al., 2016; SINGH; SINGH, 2012), de depilação (TRAVERSA; MACHADO-SANTELLI; VELASCO, 2007), para melhoramento da penetração transdérmica de medicamentos (SIM et al., 2003) e esfoliante (BANCHHOR; SARAF, 2008; HARA et al., 2014). Um importante foco para utilização de papaína, e também outras enzimas, em formulações cosméticas e farmacêuticas, está na estabilização das proteínas com matrizes poliméricas para evitar ou diminuir possíveis efeitos danosos a atividade do ingrediente ativo. Diferentes estudos já avaliaram esses fatores (HARA et al., 2014; PINTO et al., 2007, 2011; SIM et al., 2000; TRAVERSA; MACHADO-SANTELLI; VELASCO, 2007).

Uma interessante forma de estabilizar enzimas e viabilizar seu uso como esfoliante está relacionado com a imobilização de enzimas em máscaras faciais. Máscaras faciais são cosméticos que se tornaram populares nos últimos anos devido à sua facilidade de uso, custo, efetividade (AFONSO *et al.*, 2019) e por poderem trazer diversos ingredientes em sua composição. As máscaras faciais podem ser classificadas quanto ao seu modo de aplicação no rosto como folha, descamação e lavagem: enquanto a primeira é caracterizada por um material sólido ou semi-sólido de formato da face, as outras duas são aplicadas em forma de creme ou gel (NILFOROUSHZADEH et al., 2018). As máscaras tipo folha são feitas de materiais poliméricos que se moldam a face e se caracterizam por serem finas. Esse tipo de máscara pode ser produzido por diferentes materiais, mas havendo destaque para uso de biopolímeros (MORGANTI et al., 2020). As máscaras tipo de folha são as mais indicadas para uso como suporte para imobilização de enzimas.

Diferentes biopolímeros já foram avaliados para produção de máscaras faciais cosméticas. Dentre as propriedades desejadas para aplicação como máscaras faciais, estão baixo custo (devido ao uso único), flexibilidade, resistência suficiente par aplicação na face, boa

absorção de água, propriedades de barreira suficientes para evitar rápida evaporação dos ingredientes ativos, biodegradabilidade, biocompatibilidade e não-toxicidade. O amido é um polissacarídeo que tem diversas das características desejadas para esse tipo de aplicação. Ele pode ser extraído de diferentes plantas e apresenta como característica boas propriedades de formação de filmes. Além disso, o processo de gelatinização desse polímero é conhecido por tornar os filmes mais resistentes, com melhores propriedades de barreira e menos solúvel (COLTELLI et al., 2018; JHA et al. 2020; RATNAYAKE; JACKSON, 2008). Apesar de ter propriedades para aplicação como máscara facial, filmes de amido de baixo custo ainda são considerados frágeis e muito solúveis. Assim, estudos já avaliaram e comprovaram que a produção de filmes com a blenda com derivado de celulose, carboximetilcelulose (CMC), produz matrizes com propriedades mecânicas e de barreira superiores aos filmes puros de ambos polímeros (GHANBARZADEH; ALMASI; ENTEZAMI, 2010; MA; CHANG; YU, 2008; SURIYATEM; AURAS; RACHTANAPUN, 2019; TONGDEESOONTORN et al., 2011). Para diminuir ainda mais a solubilidade desses polímeros, ambos podem ser reticulados com ácido cítrico, reticulante de baixo custo e atóxico (GHANBARZADEH; ALMASI; ENTEZAMI, 2010).

As técnicas de imobilização de enzimas são realizadas por métodos de aprisionamento, reticulação, adsorção e ligação covalente. A escolha de técnica deverá levar em conta o tipo de enzima, suporte utilizado, aplicação e outros fatores (CEN et al., 2019; MOHAMAD et al., 2015; REIS et al., 2019; SHELDON, 2007). Pensando na aplicação como máscara facial, é interessante evitar o uso de técnicas e compostos que possam causar irritação, toxicidade e efeitos indesejados.

Objetivos

O objetivo principal desse estudo foi avaliar a capacidade da papaína para atuar como agente esfoliante cosmético, sendo explorada a hidrólise de proteínas da pele e a possibilidade de imobilização da enzima em filmes de amido e CMC para produção de máscara facial cosmética com propriedades ativas.

Metodologia

O presente trabalho é apresentado em quatro partes, uma de revisão bibliográfica e três etapas que envolvem aspectos experimentais. A revisão da literatura foi realizada buscando, principalmente, entender e elucidar os principais parâmetros que afetam enzimas para aplicação como esfoliantes. Ainda, foram avaliados os trabalhos realizados pela comunidade científica em diferentes bases de dados em relação ao tema nos últimos 20 anos.

Na segunda etapa, avaliou-se a atividade proteolítica de diferentes concentrações de solução de papaína (0,5 – 2,0 mg mL^{-1}) utilizando caseína, colágeno e queratina como substrato. A atividade caseinolítica (PU) foi determinada conforme Homaei et al. (2010). A determinação de atividade colagenolítica (CU) utilizou colágeno extraído de couro bovino; a reação foi realizada conforme Souchet e Laplante (2011). Por sua vez, a atividade queratinolítica (KU) foi realizada utilizando penas de peru e usando metodologia adaptada de Wu et al. (2017). A atividade enzimática com colágeno e queratina foi correlacionada com a quantificação dos aminoácidos liberados durante a hidrólise das proteínas pelo método de ninhidrina (ZHANG et al., 2013). Foi realizada a hidrólise (grau de hidrólise) das proteínas ao longo do tempo, conforme Reynaud et al. (2020) e os efeitos com relação à temperatura (23 – 37 °C) e pH (5,5 – 7,5). Por fim, ainda se avaliou a exfoliação *in vitro* de pele de porco por meio de micrografias obtidas por microscopia eletrônica de varredura. As amostras foram fixadas com tetróxido de ósmio e, então, com glutaraldeído.

Diferentemente da etapa dois (baseada no uso da enzima livre), a etapa três consistiu na imobilização de papaína em filmes poliméricos para atuarem como máscara facial. Para isso, misturou-se, proporção 1:1, 5% (m/v) de solução de amido de batata com 1 % (m/v) de CMC com auxílio de agitador mecânico. Previamente, a solução de amido foi gelatinizada a 80 °C por 30 min na presença de 0,5 % (m/v) de ácido cítrico. Diferentes concentrações de papaína foram previamente dissolvidas junto com solução de CMC: 0 – 20 % (g de papaína por g de polímero total). Então, as soluções poliméricas foram dispensadas em placas de acrílico (20 cm^2) para produção de filmes, com prévia adição de 1,15 % (m/v) de glicerol. Os filmes foram caracterizados por meio de aspecto visual, microestrutura, espessura, distribuição de proteínas, teor de umidade, solubilidade, ângulo de contato com a água, tensão de ruptura e alongamento na ruptura e composição química, essa por meio de espectroscopia no infravermelho por transformada de Fourier (FTIR). A atividade enzimática dos filmes foi realizada utilizando caseína, adaptado de Homaei et al. (2010), e pele de porco, adaptado de Gonzalo et al. (2020), como substratos. Também foi avaliada a estabilidade ao armazenamento (ANVISA, 2004).

Na quarta etapa, filmes de amido e CMC foram produzidos, similarmente à etapa três, mas sem incorporação de papaína. Então, a enzima foi imobilizada (adsorção) nos filmes (CASTRO et al., 2022; SIMÕES et al., 2021). Foram realizados testes em diferentes pHs para promover a imobilização: 6,0, 7,0 e 8,0. Também foi realizada a ativação dos filmes com adição de 0,05 – 0,20 % (m/v) de HCl, adaptado de Hanušová et al. (2013). Os filmes foram caracterizados quanto sua espessura, teor de umidade, solubilidade, ângulo de contato com a água, permeabilidade ao vapor d'água, propriedades mecânicas, composição (FTIR) e propriedades térmicas. Além disso, foram determinadas a atividade proteolítica dos filmes e parâmetros de cinética de Michaelis-Menten (HOMAEI et al., 2010).

Resultados e discussão

Os principais parâmetros e estudos realizados visando a aplicação de enzimas como agentes esfoliantes foram levantados, um fator que provavelmente servirá de base para futuras pesquisas no tema (etapa um). Vale destacar que estudos envolvendo eficiência *in vivo* e segurança ainda são escassos nesse tipo de aplicação.

Na etapa dois, observou-se que o aumento da concentração de papaína de 0,1 para 1,0 e 2,0 mg mL^{-1} aumentou as atividades caseinolítica, colagenolítica e queratinolítica. Quando 2,0 mg mL^{-1} de enzima foi utilizada, observou-se o maior valor de grau de hidrólise para caseína, colágeno e queratina: 14, 35 e 6 %, respectivamente (obtido em 240 min de reação). A adição de 3 mM de cisteína melhorou todas as atividades proteolíticas avaliadas, demonstrando o efeito de ativação desse aminoácido em relação a papaína. O uso de maiores concentrações de cisteína ou o uso de outros agentes, como sulfito de sódio, não foram capazes de melhorar a hidrólise das proteínas estudadas. A redução de pH e da temperatura acarretou a diminuição de todas as atividades proteolíticas. Papaína foi capaz de manter pelo menos 50 e 40 % de sua atividade inicial a 26 °C e pH 4,5, respectivamente. As micrografias da superfície da pele demonstraram que a aplicação tópica de enzima causou atividade de esfoliação da pele, efeito similar ao obtido com aplicação de esfoliante químico (ácido tricloroacético, 10 % (m/v)).

Os resultados da terceira etapa demonstraram que a incorporação de 10 e 20 % de enzimas forma filmes heterogêneos e com falhas, diferente das outras concentrações. A incorporação de papaína produziu filmes com matriz mais concisa visto que os filmes com 1 e 2 % de enzima obtiveram aumento nas propriedades mecânicas (tensão e alongamento na ruptura) e menor solubilidade, quando comparados ao filme controle (sem papaína). Os filmes contendo enzima apresentaram atividade proteolítica para ambos substratos utilizados, demonstrando que o processamento manteve a atividade da enzima. As micrografias das peles

tratadas com os filmes contendo papaína apresentaram efeitos de esfoliação similares àqueles observados quando a enzima foi aplicada em forma tópica. Além disso, uma boa distribuição de enzimas foi observada ao longo das matrizes poliméricas. Para finalizar a etapa três, os resultados de estabilidade mostraram que a imobilização contribuiu para uma maior manutenção de atividade, atingindo, pelo menos, 90 dias, enquanto que a enzima na forma livre perdeu a atividade em menos de 30 dias. O ambiente refrigerado (6 °C) mostrou ser a melhor condição de armazenamento, quando comparado à temperatura ambiente (22 °C) ou superior (37 °C), sendo mantida, aproximadamente, 40 % de toda atividade nessa condição no último dia de ensaio.

Na quarta etapa, observou-se que maiores concentrações de papaína poderiam ser imobilizadas nos filmes utilizando-se o processo de adsorção, quando comparado à incorporação direta na matriz polimérica. A melhor condição de pH para imobilização foi em 8,0, sendo retida 42 % de toda atividade disponível. A ativação com 0,10 % (m/v) de HCl melhorou a retenção de atividade para 68 %, enquanto que os tratamentos com 0,05 e 0,20 % do ácido não alterou e diminuiu, respectivamente, a atividade enzimática quando comparada com testes sem ácido. Esse efeito possivelmente se deu pela produção de grupos hidroxila adicionais na superfície dos filmes, o que foi confirmado pela análise de FTIR com aparecimento de novos picos referentes a esses grupos. Com relação aos parâmetros cinéticos de Michaelis-Menten, os filmes apresentaram menor velocidade máxima (v_{max}) e afinidade pelo substrato (esse avaliando pelo parâmetro k_M) quando comparados à enzima livre. Contudo, o filme acidificado previamente apresentou v_{max} e k_M mais próximos da enzima livre que o filme sem ativação. Em relação à caracterização, a maioria das propriedades acabou não sendo influenciada pelos processos de imobilização e/ou ativação. Em geral, os filmes apresentaram redução na quantidade de absorção de água e sua superfície apresentou-se mais hidrofóbica. Tanto a análise de FTIR quanto térmica demonstraram que os filmes apresentaram picos e bandas parecidos com os polímeros e enzima puros.

Conclusões

A pesquisa bibliográfica destacou as enzimas como agentes esfoliantes, enfocando os principais achados que as pesquisas científicas encontraram nos últimos anos e aspectos relacionados à tecnologia enzimática que podem interferir nesta aplicação cosmética.

Os resultados em relação ao estudo da enzima na forma livre demonstram que papaína pode ser utilizada em formulações cosméticas (e farmacêuticas) para aplicação na pele, pois a enzima é capaz de apresentar atividade de hidrolisar proteínas solúveis e insolúveis desejadas. A escolha da concentração do catalisador é importante para garantir sua eficiência. Demonstrou-se ser muito importante adicionar cisteína juntamente com a enzima para que ocorra uma melhoria na atividade enzimática. A papaína foi capaz de manter sua atividade mesmo nas condições de pH e temperatura da pele (pH 5,5 e 34 °C, aproximadamente). Por meio de ensaios *in vitro* observa-se atividade esfoliante da enzima na pele.

Os filmes de amido e CMC têm propriedades interessantes para aplicação como suportes para imobilização da papaína, e, principalmente, para atuar como máscara facial cosmética. A incorporação direta da enzima nas soluções filmogênicas (etapa três) melhora algumas propriedades dos filmes, como mecânicas e solubilidade. Além disso, os filmes são capazes de auxiliar na manutenção da atividade da enzima por mais tempo, chegando até 90 dias, quando comparada com a enzima livre. Além disso, na quarta etapa, a maior concentração de papaína (do que direta incorporação) foi imobilizada nos filmes. Ambos usos de tampão alcalino (pH 8,0) e ativação da superfície dos filmes com ácido (0,10 % (m/v) de HCl) melhoram a retenção de atividade enzimática. O estudo demonstrou que a papaína pode ser utilizada como agente esfoliante em máscaras faciais feitas de biopolímeros.

RESUMO

A papaína é uma enzima proteolítica que pode ser utilizada como agente esfoliante cosmético. Esse tipo de aplicação é baseado na hidrólise das proteínas da pele que estimula a produção de novas camadas desse órgão. Outro cosmético interessante que tem se destacado nos últimos anos são as máscaras faciais cosméticas. Esse tipo de máscara é considerado bom e efetivo e pode ser utilizado na rotina de *skincare* de pessoas. Máscaras faciais do tipo folha podem atuar como suportes para imobilização de papaína e produzir cosméticos ativos. O presente trabalho tem como objetivo avaliar a capacidade da papaína de atuar como agente esfoliante em cosméticos, explorando a hidrólise de proteínas da pele e sua imobilização em filmes de amido e carboximetilcelulose (CMC) para produzir máscaras faciais cosméticas ativas. Os filmes foram físico-quimicamente caracterizados. Primeiramente, uma revisão da literatura foi escrita para demonstrar os principais tópicos e dar um panorama geral sobre o uso de enzimas proteolíticas como esfoliantes. A atividade proteolítica de diferentes concentrações de papaína foi estudada usando caseína, colágeno e queratina como substratos. Os efeitos da cinética de curso de tempo, pH, temperatura e esfoliação in vitro foram realizados. Os filmes da blenda de amido e CMC (razão em massa 1:1) foram obtidos por casting. Diferentes quantidades de papaína foram incorporadas na solução filmogênica. Ainda, a papaína foi imobilizada por adsorção nos filmes, variando-se o pH da solução e avaliando a ativação prévia da superfície dos filmes com HCl. Os resultados mostraram que o uso de 3 mM de cisteína melhorou a atividade proteolítica da papaína. O aumento da concentração de enzima aumentou a atividade proteolítica da enzima tanto na sua forma livre quanto imobilizada. A enzima foi capaz de hidrolisar as proteínas em baixo tempo de contato. A papaína apresentou atividade em pH e temperatura na região da pele humana. A incorporação de papaína melhorou as propriedades mecânicas dos filmes e ângulo de contato enquanto outras propriedades não foram afetadas. O uso de pH alcalino e, principalmente, ativação com HCl proporcionaram as melhores condições para adsorver a papaína nos filmes. Ambas estratégias de imobilização de enzima produziram filmes ativos. Os resultados de esfoliação *in vitro* mostraram que ambas solução e filmes com papaína apresentaram padrões similares aos esfoliantes controle. Em relação à estabilidade, os filmes preservaram a atividade da papaína por pelo menos 90 dias enquanto a solução líquida reteve sua atividade por menos de 30 dias. Assim, foi demonstrado que a papaína pode ser utilizada como esfoliante enzimático e ser imobilizada em filmes como máscaras faciais ativas.

Palavras-chave: Cosméticos; Enzimas; Estabilidade; Formulação; *Skincare*.

ABSTRACT

Papain is a proteolytic enzyme that can be applied in the cosmetic sector as an exfoliant. This type of application is based on promoting the hydrolysis of the skin proteins to stimulate the production of new layers of the organ. Another interesting cosmetic that has been highlighted recently is sheet beauty face masks. These types of masks are considered good, and effective, and have been used in the skincare routine of people. Sheet beauty masks can act as supports for immobilizing papain and produce an active cosmetic. The present study aimed to evaluate the capability of papain to act as an exfoliating agent for cosmetics, exploring the hydrolysis of proteins of the skin and the immobilization of the enzyme in films of starch and carboxymethyl cellulose (CMC) to produce active beauty masks. The films were physic-chemical characterized. Firstly, a literature review was written to demonstrate the main topics and an overview of the use of proteolytic enzymes as exfoliants. The proteolytic activity of different concentrations of papain was studied using casein, collagen, and keratin as substrates. The effects of time-course kinetics, pH, temperature, and *in vitro* exfoliation were performed. Films of the blend of starch and CMC (1:1 ratio) were obtained by casting. Different amount of papain was incorporated into the filmogenic solution. In addition, papain was immobilized by adsorption in the films varying the pH of the solution and evaluating the previous activation of the surface of the films with HCl. The results showed that using 3 mM of cysteine improves the proteolytic activity of papain. Increasing the enzyme concentration increase the proteolytic activity of both free and immobilized papain. The enzyme was able to hydrolyze the proteins at low contact times. Papain presented activity in pH and temperature of the range of human skin. The incorporation of papain improved the mechanical properties of the films and the contact angle while others properties were not affected. The use of alkaline pH and, principally, activation with HCl provided the best condition for adsorbing papain on the films. Both strategies of immobilizing papain provided active films, while the adsorption enabled to immobilize more enzyme. The in vitro exfoliation results showed that both solution and films with papain presented a similar pattern to control exfoliants. Regarding the storage stability, films preserved the activity of papain for at least 90 days while the papain solution for less than 30 days. Thus, it was demonstrated that papain can be used as an enzyme exfoliant and be immobilized in films to produce beauty face masks with this property.

Key-words: Cosmetics; Enzymes; Formulation; Skincare; Stability.

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LIST OF ABBREVIATIONS AND ACRONYMS

Abbreviation/ Acronym	**Meaning**
AF	Acidified film
ANOVA	Analysis of variance
APF	Acidified papain film
APSS	Azo porcine skin substrate
Asn	Asparagine
ATR	Attenuated total reflectance
CF	Control film
CMC	Carboxymethyl cellulose
Cys	Cysteine
DSC	Differential scanning calorimetry
EDTA	Ethylenediaminetetraacetic acid
FTIR	Fourier-transform infrared spectroscopy
Gln	Glutamine
His	Histidine
P0	Film containing 0 % (w w^{-1}) of papain per total polymer in the films
P1	Film containing 1 % (w w^{-1}) of papain per total polymer in the films
P2	Film containing 2 % (w w^{-1}) of papain per total polymer in the films
P5	Film containing 5 % (w w^{-1}) of papain per total polymer in the films
P10	Film containing 10 % (w w^{-1}) of papain per total polymer in the films
P20	Film containing 20 % (w w^{-1}) of papain per total polymer in the films
TCA	Trichloroacetic acid

LIST OF SYMBOLS

Symbol	Meaning	Unit
ΔRH	Difference of relative humidity	
A	Area of the film	cm^2, m^2
CU	Collagenolytic activity	-
G	Permeation rate	$g\ h^{-1}$
IA	Immobilized activity	
IY	Immobilization yield	%
kcat	Turnover number	
k_M	Michaelis constant	mM
KU	Keratinolytic activity	-
L	Thickness	mm
MC	Moisture content	%
N	Sample number	-
$NH_2(t)$	Concentration of α-amino groups released in the mixture	mM
$NH_2(t_0)$	Initial concentration of α-amino groups released in the mixture	Mm
$NH_2(t_{total})$	Concentration α-amino groups in the mixture determined after acid hydrolysis	mM
P	Saturation vapor pressure of water	kPa
PU	Caseinolytic activity	-
R^2	Coefficient of determination	
RA	Recovered activity	%
SM	Solubility matter	%
t	Time	min, h
v_{max}	Maximum velocity	
WA	Water absorption capability	$g\ g^{-1}$
WCA	Water contact angle	°
WVP	Water vapor permeability	$g\ mm\ m^{-2}\ h^{-1}\ Pa^{-1}$
Wi	Initial weight of the film	g
Wf	Final weight of the dried film	g

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Conceptual Diagram

Why?

There is a tendency for the use of bioderived and eco-friendly compounds in cosmetics;

Proteolytic enzymes are capable to hydrolyze proteins of the skin and act as exfoliants;

There are few reports regarding the use of papain and enzymes as exfoliants;

Face masks market growth in recent years;

Sheet face masks present interesting properties to be used as supports to immobilize enzymes;

Biopolymeric-based face masks can be easily modified to immobilize enzymes.

What was already reported?

There are several cosmetic products containing enzymes that report enzymatic exfoliation activity;

Few studies reported the activity of papain in the hydrolysis of proteins of the skin;

In vivo topical application and visual evaluation of the use of solutions containing enzymes in the skin;

Films of starch and carboxymethyl cellulose have been studied and seem to have interesting characteristics to act as beauty face masks;

Different components and additive ingredients have been evaluated to produce active bio-based face

Hypotheses

Is papain capable of promoting the hydrolysis of proteins of the skin in the conditions of pH and temperature of the organ?

How long and how much papain is needed to have an exfoliating effect?

Do films of starch and CMC present the properties needed to act as beauty face masks?

Does papain maintain its enzymatic activity in polysaccharide-based films after immobilization?

Does the surface modification of films improve enzyme immobilization?

Expected results

Papain presents enzymatic exfoliation activity by hydrolyzing skin proteins in pH around 5.0 - 6.0 and 37 °C and in low contact times with the skin;

The immobilization of papain in starch and CMC films maintained the activity of the enzyme without the use of other compounds;

The surface modification of the films improved the enzyme immobilization and exfoliation activity.

STRUCTURE OF THE THESIS

This thesis was structured in chapters. Chapter 1 introduces the topics that were researched in the study and bring the objectives of the thesis. Chapter 2 describes the main topics that were researched in the literature involving the skin, skincare products, exfoliants, enzymatic exfoliation, papain, beauty face masks, biopolymeric films, and enzyme immobilization. Moreover, in Chapter 2 is detailed a review of general aspects of enzymatic exfoliation.

Chapters 3, 4, and 5 present the experimental data obtained during the study, and each one is referred to a different article. Chapter 3 refers to the study of the evaluation of papain in hydrolyzing proteins of the skin, keratin and collagen, and the standard protein, casein. In this section, the effects of papain concentration, presence of activators, time-course hydrolysis, pH, and temperature were evaluated aiming at a pre-formulation cosmetic study. Moreover, the *in vitro* effect of topical application of papain in skin sections was also studied. In this chapter, the enzyme was evaluated in the solution (free) form.

Chapters 4 and 5 were centralized in immobilizing papain in films of starch and carboxymethyl cellulose to produce an active beauty face mask with exfoliation activity. In Chapter 4, different amounts of papain were directly incorporated into the filmogenic solution. On the other hand, in Chapter 5, the enzyme was adsorbed principally on the surface of the films in an attempt to improve the enzyme concentration. In both chapters, the obtained films were physic-chemical characterized and the proteolytic activity of the obtained material was evaluated to ensure that the product maintained the activity of papain.

Finally, Chapter 6 presents the main conclusions of the work and, also, describes some suggestions for future studies involving the studied themes. All references used in the study were listed in the References section.

CHAPTER 1: INTRODUCTION AND OBJECTIVES

1.1 INTRODUCTION

Different types of skincare products have been highlighted in recent years (NAFISI; MAIBACH, 2017). Among them, exfoliants have entered into the routine of the people to improve the appearance of the skin. The exfoliants can be classified as chemical, physical (or mechanical), and enzymatic, depending on the component that is used. Physical exfoliants have been avoided because this type of product is known to cause environmental problems (microplastic) (PACKIANATHAN; KANDASAMY, 2011). Chemical exfoliation is the most common procedure, but since acids were used, the exfoliation is not specific and can degrade undesired structures of the skin (GANCEVICIENE et al., 2012). The use of enzymes is considered an excellent alternative for this type of product since there was an increase in the use of eco-friendly and bioderived products (SHOUKET et al., 2020; SUNAR; KUMAR; DESHMUKH, 2016). Despite the existence of cosmetics containing enzymes (SIM et al., 2000; SUNAR; KUMAR; DESHMUKH, 2016), studies regarding enzymatic exfoliation have been neglected in the scientific literature.

Enzymatic exfoliation is based on mimicking the natural renewing process of the skin, the desquamation. Briefly, proteolytic enzymes were applied to the surface of the skin to promote the hydrolysis of the proteins of the skin (HARA et al., 2014; LANGER et al., 2013; PINTO et al., 2011). This action causes a cascade effect, accelerating the metabolism to produce new layers of the skin and, consequently, reaching for the “new” and “healthy” appearance of the organ (EGELRUD, 2000; PACKIANATHAN; KANDASAMY, 2011). Different proteolytic enzymes can be used as exfoliants, but preferably the ones that present activity in the temperature and pH of the skin and that can hydrolyze proteins of the skin (GONÇALVES, 2021).

Papain (EC 3.4.22.2) is a plant-based cysteine protease extract from the latex of *Carica papaya*. Papain is the most studied and industrial used protease (FERNÁNDEZ-LUCAS; CASTAÑEDA; HORMIGO, 2017). For this reason, this enzyme is used for several purposes. From the food and brewing industries to pharmaceutical and cosmetics. In the last two types of application, papain can be used as a debridement agent of wounds (LANGER et al., 2013; PORSANI et al., 2016; SINGH; SINGH, 2012), transdermal drug enhancer (SIM et al., 2003), depilatory agent (TRAVERSA; MACHADO-SANTELLI; VELASCO, 2007), and exfoliant

(CHAVAN, 2015; HARA et al., 2014; SIM et al., 2000). Its uses in the skin are related to the papain present wide-range activity from different proteins and operational stability of pH and temperature (FERNÁNDEZ-LUCAS; CASTAÑEDA; HORMIGO, 2017).

One of the main problems related to the application of enzymes in cosmetics is related to the loss of activity that can occur depending on the formulation. Some studies have focused on complexing the enzymes in polymeric matrices to improve the stability of the active ingredient (HARA et al., 2014; PINTO et al., 2007, 2011; SIM et al., 2000; TRAVERSA; MACHADO-SANTELLI; VELASCO, 2007). Aiming the enzymatic exfoliation, and the immobilization of enzymes in beauty face masks seems to be interesting. In this application, the product stability can be achieved simultaneously with the application direct in the desired area.

Enzyme immobilization is the process that is performed to attach an enzyme to a material, named support. Depending on the type of immobilization technique used, enzymes can be physically or chemically, reversibly or irreversibly, immobilized in a support. The techniques of enzyme immobilization are divided into entrapment, adsorption, crosslinking, and covalent bonding (REIS et al., 2019; SHELDON, 2007). The choice of technique depends on the enzyme, support, application, etc. Generally, the support can be functionalized and/or activated to improve immobilization yield and the recovered activity. It is important to not use the active site of the enzyme to immobilize the protein to avoid loss in the activity of the enzyme (MOHAMAD et al., 2015).

Beauty face masks are cosmetics that were used to cover the face and are known to be easily used and present fast and high effectiveness (PERUGINI et al., 2019). Face masks can be classified as sheet, wash-, and peel-off, depending on the type of material used to produce them. Face masks were used to moisturize the skin, avoid the rapid evaporation of other cosmetics used together with this product, and can be incorporated with several active ingredients, depending on the objective, as exfoliants. Sheet masks are thin fabrics, polymeric films, or hydrogels, that cover the skin. Different shapes and structures can be used (NILFOROUSHZADEH et al., 2018). Since they are single-use, the is a tendency for the use of biodegradable materials to produce this type of cosmetics. Biopolymeric films are interesting and promising candidates to produce sheet face masks, principally due to biodegradability added to the biocompatibility and non-toxicity properties that these polymers can present (MORGANTI et al., 2020).

Low-cost and good film-forming biopolymers are preferably for this type of application. Starch is a polysaccharide that presents both characteristics (COLTELLI et al., 2018; JHA et al., 2020). Another interesting biopolymer to be used in the same scenario is carboxymethyl cellulose (CMC). CMC is an anionic cellulose derivative that contains carboxymethyl groups partially substituting hydroxyl groups of the cellulose backbone. Films of CMC are known to be flexible and present good water retention (TREVISOL et al., 2019). Moreover, both polymers can be blended. Some studies already showed that the incorporation of CMC improved mechanical and barrier properties of starch films, for example (GHANBARZADEH; ALMASI; ENTEZAMI, 2010; MA; CHANG; YU, 2008; SURIYATEM; AURAS; RACHTANAPUN, 2019; TONGDEESOONTORN et al., 2011).

1.2 MAIN OBJECTIVE

The main objective of this thesis was to evaluate the capability of papain to act as an exfoliating agent of cosmetics, exploring the hydrolysis of proteins of the skin and the immobilization of the enzyme in films of starch and CMC to produce active beauty masks.

1.3 SPECIFIC OBJECTIVES

I. Evaluate, through a critical review, the enzymatic exfoliation, showing the main studies and aspects related to this topic;
II. Assess the influence of papain concentration, presence of activators, pH, and temperature in the hydrolysis of casein, collagen, and keratin;
III. Study *in vitro* exfoliation activity of the enzyme using skin specimen;
IV. Produce films of starch and CMC and evaluate it properties to apply as beauty masks;
V. Evaluate the incorporation of different amount of papain in films of starch and CMC and its effects on physic-chemical properties of the materials;
VI. Verify if the films presented proteolytic activity and the storage stability of the formulations;
VII. Evaluate adsorption strategies for immobilizing papain in films of starch and CMC, principally effects of pH, plasma treatment and acid activation;
VIII. Determine both free and immobilized papain Michaelis-Menten parameters and the immobilization yield, and recovery activity in the enzyme adsorbed in the films.

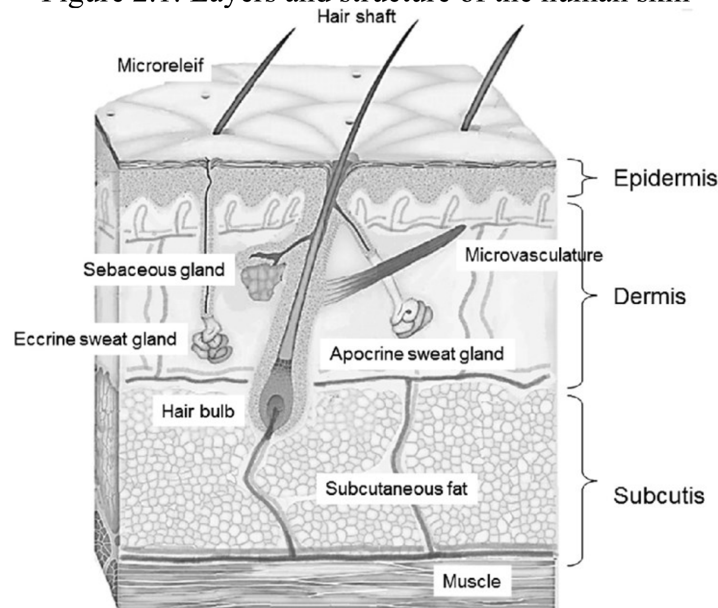
CHAPTER 2: LITERATURE REVIEW

2.1 BASIC SKIN STRUCTURE

The skin is the main organ of the human body concerning size and appearance. The beauty of the skin is considered an important and significant index of the well-being of a person. Generally, undesired visual changes in the skin tend to promote psychosocial impact in humans. Among these changes, aging is known to be the most feared cause of the variation of the skin. Moreover, other issues and diseases can affect the appearance of the skin, such as dermatitis, infections, allergies, wounds, scars, pigmentation changes, etc. (BAYAT; MCGROUTHER; FERGUSON, 2003; CAVINATO, 2018; FONACIER; DRESKIN; LEUNG, 2010; KANJI, 2019).

The skin corresponds to 16% of human weight and its total area measures, in adults, between 1.6 and 1.8 m^2. This organ has various functions, such as barrier and infection protector, promoter of homeostasis maintenance, and plays a role in the synthesis of vitamin D. The skin is divided into two main-layers, epidermis and dermis, and in contact with a third layer, hypodermis (FENNER; CLARK, 2016). Figure 2.1 shows the structure of the skin with the different layers.

Figure 2.1: Layers and structure of the human skin



Source: Hirao (2017).

The hypodermis is made of fatty connective tissues and not necessarily is considered a layer of the skin. However, due to its location, hypodermis is generally associated with this organ. The main role of hypodermis is to maintain the dermis layers above the muscles and bones and regulate the temperature. This layer is composed of adipose lobules along with different skin appendages like the hair follicles, sensory neurons, and blood vessels (BARBIERI; WANAT; SEYKORA, 2014; MONTEIRO-RIVIERE, 2010).

The dermis-epidermis junction is a complex and specialized structure that regulates the signals of the proliferation and differentiation of keratinocytes. After this junction, there is the dermis. This layer of the skin is also subdivided into different parts: the superior (papillary) and the inferior (reticular). The papillary layer contains to maintains the connection between the dermis and epidermis. The reticular layer is composed of collagen (type I and IV) and elastin. Both proteins are immersed in a fluidic structure named ground substance, which is made of glycosaminoglycans (GAG). On the other hand, GAG is composed of different polysaccharide chains, such as hyaluronic acid, that are responsible to maintain the connection with water molecules and improve intercellular communication and the dermis stability (LEES, 2012; SHOULDERS; RAINES, 2009).

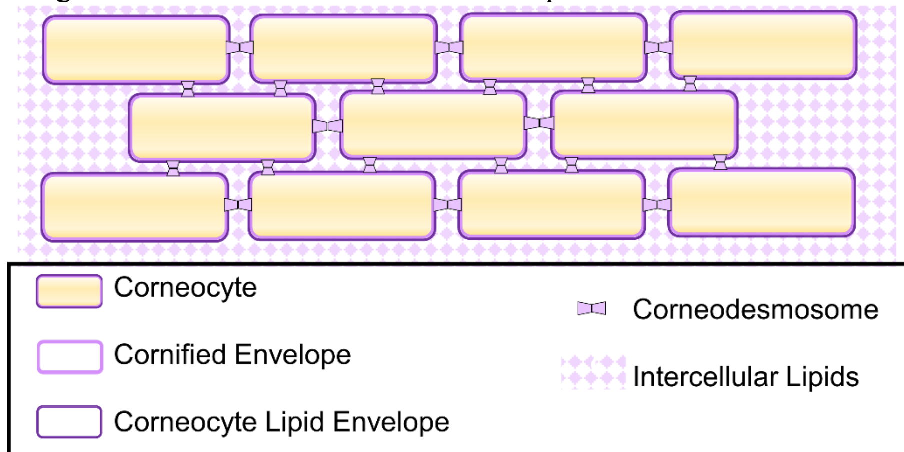
The epidermis is the most external and thin layer of the skin. The epidermis is segmented into distinct stratums, which one with different structures and functions (MONTEIRO-RIVIERE, 2010). The outmost stratum is the stratum corneum. The stratum corneum is constituted by different layers of flattened corneocyte cells. The corneocytes are enucleated cells that are considered devitalized. Below the stratum corneum, there is the viable epidermis. The viable epidermis is composed of different layers from outside to inside: stratum lucidum, stratum granulosum, stratum spinosum, and stratum basale. The term viable is used to demonstrate that, differently from the stratum corneum, this region is biochemically active. Keratinocytes (at different levels of differentiation), melanocytes, Langerhans cells and Merkel cells are found in the viable epidermis (HIRAO, 2017; MENON; CLEARY; LANE, 2012).

The cosmetic segment develops techniques and products to attempt to prevent or minimize the effects of appearance and function changes of the skin. For this reason, different researches are focusing in formulations to penetrate and act into and through the stratum corneum (KIM et al., 2020).

2.1.1 Stratum corneum

The stratum corneum has 10 – 20 µm of thickness, The brick-and-mortar model is utilized to represent the stratum corneum and is shown in Figure 2.2. The bricks are the corneocytes and the mortar is the intercellular space made of lamellar lipid bilayers that surrounds the cells (HIRAO, 2017). The corneocytes are keratinocytes that have differenced and lost the nucleus, intercellular organelles, and plasmatic membrane (BOSKO, 2019). The lipid bilayer represents 15% of the total weight of the stratum corneum and it is composed mainly of ceramides (50%), cholesterol (25%), and free fatty acids (10 – 20%), etc. (HARDING, 2004).

Figure 2.2: "Brick and mortar model" representation of stratum corneum.



Source: Trevisol et al. (2021).

The corneocytes contain keratin macro fibrils stabilized by disulfide bonds and are surrounded by the cornified envelope (HARDING, 2004). Keratin is the protein that forms the cytoskeletal fiber network of the skin cells and corresponds to 80% of the total protein content of the stratum corneum (HIRAO, 2017). Beyond the presence of keratin, the corneocytes are also composed the natural moisturizing factors (NMF). NMF are formed by small hygroscopic molecules such as amino acids, organic acids, and mineral salts, that can retain water in the stratum corneum (HIRAO, 2017). The cornified envelope is composed of crosslinked proteins (involucrin, loricrin, and proline-rich proteins) and lipids that interact with the intercellular lipids (WOLK; WITTE; SABAT, 2010).

The corneocytes were connected by adhesive protein structures, the corneodesmosomes. The external part of the corneodesmosomes is composed of two glycoproteins, the desmosome cadherins: desmoglein-1 and desmocolin-1 (ISHIDA-

YAMAMOTO; IGAWA, 2015). A third protein is responsible to increase cell-cell adhesion, the corneodesmosin (BOSKO, 2019). The degradation of the external structure of the corneodesmosin comprehends the desquamation process of the renewing of the skin (ISHIDA-YAMAMOTO; IGAWA, 2015).

2.2 SKINCARE COSMETICS

The appearance of the skin is an important parameter for a person's well-being. Beyond the barrier function, the skin is a social communicative organ. When the stratum corneum is dry, rough, unappealing to touch, or presents other undesired appearance, this may induce psychosocial problems (LODÉN, 2014). Cosmetics are agents that meet an important psychosocial-biological need of the human being to feel good in an attempt to maintain or improve the structures, functions, and appearance of the body (RATTAN, 2015).

There is a wide variety of different cosmetic products. The presence of different cosmetics is directly related to the growth of the section in the past years. The global sales of the beauty industry increased from US$ 15 billion in 2005 to US$ 21 billion in 2014. The sales of beauty products get a boost even during the global economic decline of the Great Recession (2000 – 2010). Because beauty seems to be recession-proof, Leonard Lauder, then president of the North American cosmetics company Estée Lauder®, nominated the expansion of the cosmetic sector as the lipstick index. The lipstick index suggests that even during crises or moments of economic difficulties, women buy lipstick (and other products) to maintain appearance, status, and feel good. Other segments, such as clothing, footwear, and jewelry, did not observe this behavior (MACDONALD; DILDAR, 2020).

Despite the resilience of the beauty industry, the COVID-19 pandemic affected this sector. But the recession in this sector was lower than in other segments. As people stayed at home for a long period and had to wear protective devices, such as masks, makeup products had their use decreased (GERSTELL et al., 2020). On the other hand, do-it-yourself and skincare products gained attention and use during the pandemic (SANCHEZ, 2020; SCHIFFER, 2020). As Covid cases fell, people started to be encouraged to rediscover their social lives and, consequently, come back with cosmetic use habits. It is estimated that the skincare products market size to surpass US$ 200 billion by 2030 (PRECENDECE RESEARCH, 2022).

The cosmetics can be classified as skincare, haircare, coloring agents, fragrances, general care products (CAVINATO, 2018), nail care, and lip care (NAFISI; MAIBACH, 2017). Skincare concept is not well defined and can englobe all products and procedures that maintain the skin in "good condition" and clean, perfumed, protected, etc. (SURBER; KOTTNER, 2017). In this study, the terms cosmetic and cosmeceutical (products of intersection between cosmetics and pharmaceuticals) will not be distinguished due to the non-formal differentiation of these two products by regulatory agencies in most countries.

Different types of cosmetics have been developed to meet consumer demands regarding skin care (NAFISI; MAIBACH, 2017). The most used cosmetics are moisturizers, sunscreens, cleansers, and products that contain active ingredients, such as antioxidants, retinoids, exfoliants, etc. (BERMANN, 2007; CAVINATO, 2018). There are different ways to apply a cosmetic on the skin. The formulations can be topically applied as creams, gels, or solutions/emulsions or, more, being incorporated into facial masks or other vehicles, for example (NILFOROUSHZADEH et al., 2018). In the case of cosmetics containing active ingredients, it is important to ensure that the molecules maintain their activity when incorporated into the formulation and with the concentration used (BAREL; PAYE; MAIBACH, 2010; CAVINATO, 2018).

2.2.1 Exfoliants

The intrinsic capability of the skin to regenerate and produce new layers of cells, which bring the "new" aspect to the organ, is named desquamation. Desquamation is a continuous, invisible, and biologically complex process that is related to the final step of the differentiation of keratinocytes (MILSTONE, 2004). Tipically, the desquamation occurs for 2 to 4 weeks (EGELRUD, 2000) and is dependent on diverse intrinsic (age, healthy, etc.) and extrinsic agents (temperature, clothing/protection of the skin, clime, etc.) (PACKIANATHAN; KANDASAMY, 2011). Aging is known to reduce the desquamation rate of the skin: the intercellular layer that maintains the cells of the skin close to each other ends up becoming denser, which makes natural exfoliation difficult (EGELRUD, 2000; PACKIANATHAN; KANDASAMY, 2011).

The exfoliation or peeling is a procedure/product that can remove defined layers of the skin, accelerating and stimulating the desquamation. Then, a soft, firm, uniform, and glowing skin is formed (GANCEVICIENE et al., 2012; NAFISI; MAIBACH, 2017). An exfoliating

agent can be classified as superficial, when only act in the epidermis, medium, reaching the papillary layer of the dermis, and deep, when the reticular dermis is affected. On the other hand, the depth of an exfoliant acts is depended on the administration method, type of the skin, concentration and type of active ingredients, pH, etc. (LANDAU, 2008). The exfoliation agents can be classified as chemical, physical (or mechanical), or enzymatic (PACKIANATHAN; KANDASAMY, 2011).

Physical exfoliation consists of the mechanical abrasion of the skin. The friction caused by the abrasive assists in the removal of corneocytes from the corneum stratum layer. Polymeric materials were the most common abrasive agents used in cosmetics, principally microbeads made of polyethylene, polypropylene, or polymethacrylate. However, in 2018, the United States prohibited the use of microbeads in cosmetics due to the environmental problems with discarding this type of material. Similar prohibition was also observed around the world. Thus, natural alternatives have been researched, such as the use of clays, grains, seeds or plant bark, cellulose, wax, or glucomannan beads, for example (BAREL; PAYE; MAIBACH, 2010; DEHAVEN, 2015; KITSONGSERMTHON et al., 2017; PACKIANATHAN; KANDASAMY, 2011).

The chemical exfoliation is based on the use of alpha-, beta- and poly-hydroxy acids. The use of these substances is dated from Ancient Egypt with the application of sour milk (lactic acid) on the skin. However, it was only in the 1900s that dermatologists began to assess the effects on the skin of substances such as phenol, salicylic acid, and trichloroacetic acid (BRODY et al., 2000). Historically, the use of chemical exfoliants had two phases: the first is related to the discovery of substances that can present exfoliation; the second phase demonstrated the clinical evaluation and histological effects of the use of the exfoliants as well as the possible formulations and mixtures (LANDAU, 2008). Table 2.1 presents the different chemical peels and their action.

The exact mechanism of chemical exfoliant action is not elucidated. The superficial exfoliation increases the activity of the endogenous proteolytic enzymes of the skin, these enzymes are responsible to perform the epidermolysis and skin desquamation. The medium-depth exfoliants promote the coagulation of membrane proteins and destroy the epidermis and dermis cells. Lastly, the deep exfoliants coagulate the proteins of the skin, causing complete epidermolysis, reconstruction of the basal layer, and restoration of the dermis architecture (GANCEVICIENE et al., 2012).

Table 2.1: Chemical exfoliation and skin areas affected.

Depth of the exfoliation	Area of skin	Healing time	Chemical peels*
Superficial (0.06 – 0.45 mm)	Epidermis (corneum stratum to basal stratum)	3 – 5 days	Glycolic acid (30 – 70%) Retinoic acid (10%) Salicylic acid (20%) Trichloroacetic acid (10 – 25%) Jessner solution Dry ice
Medium (0.6 mm)	Papillary dermis	More than one week	Glycolic acid (> 70%) Trichloroacetic acid (35 – 50%) Jessner solution + trichloroacetic acid (35%) Glycolic acid (70%) + trichloroacetic acid (35%) Trichloroacetic acid (35%) + dry ice
Deep (0.8 mm)	Reticular dermis	More than two months	Phenol Croton oil Trichloroacetic acid (> 50%)

*: The concentration corresponds to that used in formulations. Source: (LANDAU, 2008; RENDON et al., 2010; VELASCO et al., 2004; YOKOMIZO; BENEMOND; MO, 2013).

2.2.2 Enzymatic exfoliation

This section is based in the article entitled "An overview of the use of proteolytic enzymes as exfoliating agents" that was related to this thesis. This article was published in Journal of Cosmetic Dermatology, 2022;21:3300–3307 (doi.org/10.1111/jocd.14673). According to Wiley subscription rules, the authors retain the right to include the article in a thesis, provided it is not published commercially.

2.2.2.1 Enzymes used as exfoliating agents

Enzymatic exfoliation can be used as an alternative to chemical exfoliation for people with sensitive skin, as well as for all skin types and tones (dark racial-ethnic group of people, skin phototypes IV to VI Fitzpatrick, have concerns regarding chemical exfoliation complications (CONCEIÇÃO; ADRIANO; LIMA, 2018), and to the treatment of diseases (PACKIANATHAN; KANDASAMY, 2011). The use of enzymes assists in the slight dilation of pore openings of the skin, thus facilitating the extraction of the clogged pores, comedones, and other impactions. The enzymatic exfoliation treatment is considered gentle. Enzyme exfoliants can be topically applied on the skin using fruits that contain proteolytic enzymes, like

papaya, pineapple, pomegranate, etc., or with enzymatic powders or solutions/creams (LEES, 2012).

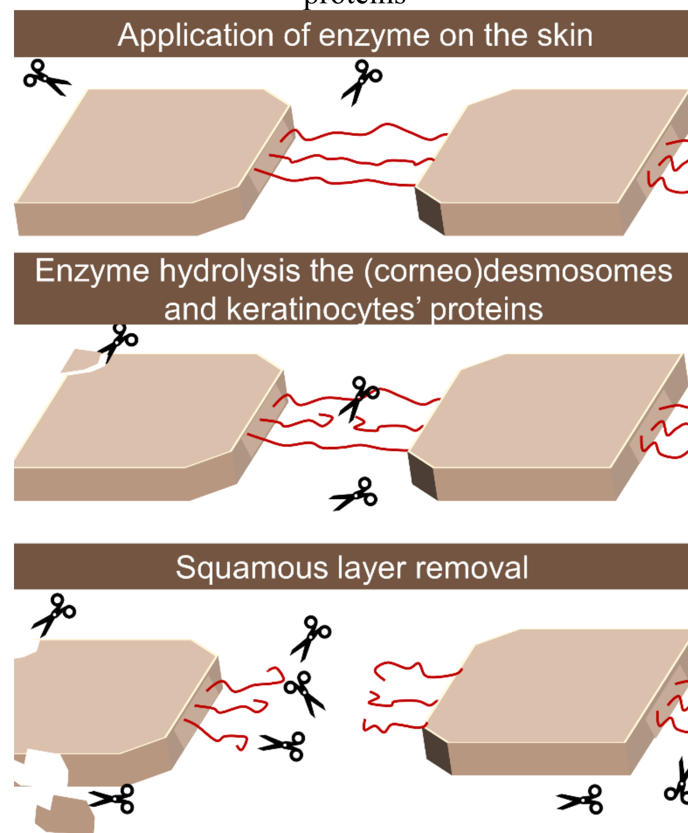
Comparing enzymatic exfoliation and other exfoliation techniques is not simple since both procedures can promote a cascade effect in the skin and have similar effects. The enzymatic exfoliation is considered gentle, the enzymes have specificity to act only in desired structures than the acids, they are bio-derived and eco-friendly (CHAVAN, 2015; FEIN et al., 2005; HARA et al., 2014; SUNAR; KUMAR; DESHMUKH, 2016), this treatment is recommended to all skin types, and it is theorized that the reduction of pH due to acid peel also promotes regulation of endogenous enzymes of the skin, promoting a combinatory effect (SMITH et al., 2007). In addition, the enzymes are pH and temperature dependents which can be used to inactive them when needed. The cons of the use of exfoliants of enzymes are: there are no sufficient data in the literature to ensure their safety, these catalyzers can be irritant or cause adverse reactions (STREMNITZER et al., 2015) (needing stabilization), and the formulation of products that contain enzymes are more difficult since some compounds can inactive or reduce the activity of these proteins.

The choice of an enzyme in an exfoliant formulation must take into account some aspects. The enzymatic exfoliation is based on the desquamation process, i.e., the enzymes used as exfoliants must catalyze the hydrolysis of the desired proteins of the skin, like keratin, desmosomes, collagen, and elastin, mimicking the desquamation. Figure 2.3 illustrates an enzymatic exfoliation process on the stratum corneum layer. Moreover, the enzyme must present catalytic activity in the desired pH and temperature of the skin. Generally, the enzymes used in cosmetics already present and established production and are researched for different applications. This occurs due to the reduction of the production costs and the need for studies to understand the properties of the enzymes. On the other hand, another strategy for choosing enzymes that act as an exfoliant is to use enzymes that present structure and function similar to endogenous enzymes that act in desquamation (VERMA; DIXIT; PANDEY, 2016) (SMITH et al., 2007; SMITH; BISHOP; NORTON, 2009).

Keratinases are the most outstanding enzymes that act in the keratin proteins. However, keratinases are, generally, more active in alkaline media and tend to have optimum activity at temperatures above 40 °C (FRIEDRICH et al., 2005; GUPTA; RAMNANI, 2006). Proteinase K, trypsin, and papain are other proteases with keratinolytic activity, even though this is not as prominent as the keratinases (JIN et al., 2017). The use of enzymes that hydrolyze desmosomes is recommended. However, there is no standard enzymatic activity of this type of

protein or a list of enzymes that present high activity against this structure. El-Kadi et al. (2001) demonstrates that the influence of enzymes in desmosomes can be observed by transmission electron microscopy of skin fragments, for example.

Figure 2.3: Enzymatic exfoliation model on the keratinocytes and (corne)desmosomes proteins



Source: Trevisol et al. (2021).

Deeper exfoliation may require hydrolysis of other proteins than keratin. Collagen and elastin are two proteins that are found in the dermis layer of the skin. Both collagen and elastin also present different types and form fibers, networks, or can be associated with other structures (FERRARO; ANTON; SANTÉ-LHOUTELLIER, 2016). Beyond collagenases, trypsin, chymotrypsin, pepsin, alkalase, properase E, pronase, papain, and bromelain are examples of enzymes capable of catalyzing the degradation of collagen (GOMEZ-GUILLEN et al., 2011). Similarly, the catalysis of the reaction hydrolysis of elastin can be promoted by elastases, papain, ficin, trypsin, and chymotrypsin (SMITH; BISHOP; NORTON, 2009).

Table 2.2 summarizes an overview of the application of enzymes in exfoliants with a similar application of the enzymes in scientific works. The research demonstrated that enzymatic exfoliation has been neglected: only eleven works were found in different databases.

The studies of enzymatic exfoliation can be divided into three categories: use of well-known enzymes and search for stabilizers, production of proteases and their catalysis of proteins from a human source, and *in vivo* studies that applied the enzymes evaluated their effects in humans.

Table 2.2: Overviewing the application of enzymes as exfoliants (or with similar use) from researches over the last 20 years.

Enzyme	What was evaluated	Main results	Reference
Papain	Stabilization of the enzyme with different polymers (dextran, glucan, or polyethyleneglycol). *In vivo* evaluation of the topical application of a solution of 1% (w/v) of papain (with polymer). Comparison with 5% (w/v) of lactic acid.	Glucan provided better results as a stabilizer. 6 daily applications of papain with glucan provided better stratum corneum removal than the acid. No severe irritation or redness was reported.	(SIM et al., 2000)
Chymotrypsin Papain Alcalase (Optimase®) (from *Bacillus licheniformis*)	Topical application of a solution of the enzymes *in vivo* (human) for 3 h. Application of moisturizing cream after the exfoliation *In vitro* (pig skin) exfoliation tests. *In vivo* (human) induction of xerosis and treatment with enzymatic exfoliation.	Visually all enzymes were able to exfoliate the skin, but alcalase provided better results. The use of moisturizing improved the skin aspect after exfoliation. The enzymes were capable of exfoliating the porcine skin with a preference for the desmosome than other proteins. The xerosis was treated, and the enzymes improved the visual aspect of the human skin.	(EL-KADI et al., 2001)
Two extracellular proteases from *Kytococcus sedentarius*	Evaluation of degradation of keratin extracted from foot callus.	Both enzymes were able to hydrolysis the keratin without any previous treatment. The proteases presented keratinolytic activity at low temperatures and pH (nearly of the skin).	(LONGSHAW et al., 2002)
Keratinase (*Doratomyces microsporus)*	Evaluation of keratinolytic activity using keratin obtained from stratum corneum of the human sole (foot). *In vitro* degradation of porcine skin epidermis in aqueous medium with a solution of 0.5% (w/v) of the enzyme.	Maximum hydrolyzed keratin was achieved after 6 h of incubation and pH 7.0. After 3 h of action *in vitro*, it was observed a slight detachment of stratum corneum from the epidermal layers. Prolonged incubation (6 and 24 h) caused almost complete separation at the epidermal-dermal level and detachment of the stratum corneum.	(FRIEDRICH et al., 2005)

Table 2.2: Overviewing the application of enzymes as exfoliants (or with similar use) from researches over the last 20 years.

Enzyme	What was evaluated	Main results	Reference
Keratinase (*Doratomyces microsporus)* (*Paecilomyces marquandii)* Elastase Trypsin Chymotrypsin Subtilisin Proteinase K Collagenase	Evaluation of keratinolytic activity using keratin obtained from stratum corneum of the human sole, keratin from human nail and hair, porcine nail, chicken feather, sheep wool, and commercial bovine keratin by the keratinases. Influence of reducing agents on the keratinase's assays. Comparison of the activity of keratinases with other proteases on stratum corneum hydrolysis.	The human stratum corneum was easily hydrolyzed, followed by bovine keratin and the nails (30 min). The wool and feathers were only hydrolyzed after 24 h of incubation. β-mercaptoethanol (5 or 25 mM) and dithiothreitol (1 or 5 mM) improved the hydrolysis. Proteinase K, *P. marquandii,* and *D. microsporus* keratinases, in this order, hydrolyzed keratin more extensively than the other enzymes.	(GRADIŠAR et al., 2005)
Dispase Trypsin	Topical application of a solution of both enzymes (0.25 and 2.5% (w/v) on hairless mice skin (*in vivo*). *In vitro* assays using human skin (from cancer surgeries) were incubated during 1, 4, and 24 h with trypsin (0.025, 0.25, and 2.5% (w/v)) or dispase (0.1 and 1.0% (w/v)).	*In vivo assays:* the solution 0.25% (w/v) did not present effects. Trypsin extensively lowered the epidermis thickness without damaging the dermis. Dispase had a lower and more located effect. *In vitro* assays: trypsin resulted in rapid intraepidermal acantholysis and dermal-epidermal separation. After 24 hours of incubation, there was complete epidermal dissolution, and the dermis was intact. Dispase resulted in purely subepidermal dissociation of intact epidermal sheets. The degree of effect was directly proportional to enzyme concentration and exposure time.	(FEIN et al., 2005)
Mucor miehei protease	Topical application of enzyme solution (15% (w/v), 3 g/cm^2) twice *in vivo* (human) in the skin followed by the use of a moisturizing agent. Evaluations of stratum corneum removal, skin hydration level, firmness, and thickness after product use.	Stratum corneum cell removal was improved by 33%, and the hydration more than 20% than the control. General skin firmness was improved by 20%. After 3 months of application, there were 12 and 17% epidermal and dermal thickness reductions, respectively, when using the protease.	(SMITH et al., 2007)

Table 2.2: Overviewing the application of enzymes as exfoliants (or with similar use) from researches over the last 20 years.

Enzyme	What was evaluated	Main results	Reference
Papain	Evaluation of skin degradation by topical application of 0.2% (w/v) of the enzyme (with cysteine) *in vitro* in human skin during 24 and 48 h.	24 h after contact with enzyme: digestion of extracellular components of corneodesmosomes and separation of corneocytes. 48 h of digestion, the outer edges of corneocytes were leached, but the cell adhesion was still maintained. After 48 h it was observed that the stratum corneum recovered by unknown mechanisms.	(LOPES et al., 2008)
Mucor miehei protease	*In vivo* (human) once a day topically application of enzyme solution (1 to 30% w/v) or lactic acid (8 and 15% w/v) evaluating different properties of the skin.	Increasing enzyme concentration reduced stratum corneum replacement time by 24 and 39% when using a solution of 25 and 30% of the enzyme, respectively (the results were similar to lactic acid). Both lactic acid and protease affected skin barrier functions, however only the patients with acid exfoliation observed skin flakiness and visible dryness. Both exfoliators have similar rejuvenation effects (skin's firmness and facial lines and wrinkles).	(SMITH; BISHOP; NORTON, 2009)
Papain	Stabilization of papain on Carbomer and alginate. Immobilization of papain with N-Hydroxysuccinimide (NHS) and 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC). Evaluation of the enzymatic activity of the complex for 6 weeks at 4, 25, and 45 °C. *In vivo* (human) evaluation of exfoliation with topical application of the complex with 1 and 3% (w/v) of the enzyme.	The complex improved the stabilization of papain activity for 12 weeks, principally at 4 °C comparing to papain free solution. 1% and 3% crosslinked papain-polymer complex provided better exfoliating activity when compared to 0.2 and 2% (w/v) of salicylic acid, respectively. The patients that used the enzyme complex related improvements in skin smoothness, roughness, and general aspects after 14 days.	(CHAVAN, 2015)

Table 2.2: Overviewing the application of enzymes as exfoliants (or with similar use) from researches over the last 20 years.

Enzyme	What was evaluated	Main results	Reference
Protease of hydrolyzed roe cream (with contain enzymes)	*In vivo* (human) twice daily topical application of hydrolyzed roe cream, 4% (w/v) glycolic acid cream or 8% (w/v) glycolic acid plus 2% (w/v) citric acid cream on the face during 12 weeks of treatment.	The hydrolyzed roe cream reduced the dyschromia counts, with improvements on brown spots. Both hydrolyzed roe cream and 8% glycolic acid plus cream improve the skin clarity and laxity parameter after 8 and 12 weeks of treatment. Only the 4% glycolic acid cream was able to reduce the effects of facial lines and wrinkles. The enzymatic cream provided less stinging and burning effect than the other products.	(MEKAS et al., 2015)

2.2.2.2 Clinical trials and safety of enzymatic exfoliation

The available clinical trials studies are not conclusive since most of the studies did not specify the numbers of test subjects or if, at least, double-blind experiments were conducted, and they only focused on simple visual observation of the skin before and after the exposure of enzymatic exfoliation. From Table 2.2, only three articles performed these aspects and are discussed below.

Smith et al. (2007) demonstrated that the use of enzymatic treatment (Cathepsin D-like enzyme) had cumulative benefits and improved both surfaces, epidermal and dermal layers of the skin compared to a placebo group. The experiments were conducted evaluating stratum corneum cell removal, skin hydration, firmness, and thickness before and after the control, enzyme, and placebo.

Smith, Bishop, and Norton (2009) compared the enzyme (Cathepsin D-like enzyme) and acid treatment (lactic acid). The best results were observed with 15 or 30% of enzyme and 8 and 12% of acid. The enzymatic and acid peel formulation presented similar results related to skin renovation time, skin hydration, and visual aspect (reduction of facial lines and age assessments). The most difference was observed in the barrier function. Both enzymatic and chemical peel application increased the transepidermal water loss rate after 4 days of treatment. However, the enzymatic exfoliation provided better recovery of barrier function, an effect not observed even after 9 days of application of the lactic acid peel. This result demonstrates that both formulas had similar action, but the enzyme one provided lower induction of dryness and flakiness.

In another study Mekas et al. (2015), the subjects were divided into groups treated with the formulation containing enzyme and compared to groups that applied acid peels. From the analysis of the images after the application of each group, whereas the acid treatment reduced superficial fine lines and the skin roughness and hyperpigmentation, the enzymatic treatment improved skin clarity and reduced photodamaged spots.

One of the major concerns of enzymatic exfoliation is related to possible side effects (CHAVAN, 2015). Topical application of enzymes in the skin can irritate or sensitize this organ, providing some side effects related to respiratory allergens and dermatitis (CHAVAN, 2015; EL-KADI et al., 2001; SMITH et al., 2007). For example, Stremnitzer et al. (2015) studied the influence of papain on the skin and demonstrated that this enzyme could degrade undesired Tight Junction (TJ) proteins.

Due to the catalytic nature and different sources, the evaluation of the safety of enzymes in cosmetics products is not simple. From the few studies that performed clinical trials of applying enzymes as exfoliants in the skin, none subject presented any adverse reaction (CHAVAN, 2015; EL-KADI et al., 2001; MEKAS et al., 2015; SIM et al., 2000; SMITH et al., 2007; SMITH; BISHOP; NORTON, 2009). However, the number of subjects evaluated in these studies did not exclude the need to conduct risk assessment evaluation. Del Rosso (2013) reviewed some aspects of the general application of proteases in dermatological products. The author suggested that additional clinical responses are needed to improve the understanding of enzyme application in the skin. Moreover, the possible interactions of other compounds of the formulation with the enzyme and also vehicle and stabilizer itself can contribute to clinical safety.

2.2.2.3 Factors that influence enzymatic exfoliation

The enzymes are dependent on physic-chemical conditions to be able to present catalytic activity. Temperature, pH, presence of activators and inhibitors, substrate specificity, enzyme concentration, and activity are important aspects to be highlighted to ensure that an enzymatic reaction occurs. In general, enzymology studies evaluate these parameters when applying this type of catalyst in reactions (WORTHINGTON; WORTHINGTON, 2016). However, as shown in Table 2.2, these parameters were generally ignored when evaluating enzymes in exfoliation products/procedures.

2.2.2.3.1 Proteolytic enzyme inhibitors in the skin

Enzyme inhibitors are molecules that can decrease or inactivate enzyme activity. Naturally, the skin has protease inhibitors to maintain equilibrium and control the action of the endogenous enzymes of this organ. However, exogenous enzymes also maintain contact to skin, either from desired origins (as an exfoliant or in other products, for example), natural microbial flora of non-host origin, derived cells (from inflammation) or undesired sources, as allergens (pollen, dust) or bacteria, fungi, and virus. In this sense, protease inhibitors can act as a barrier to prevent the effect of different proteases (MEYER-HOFFERT, 2009). The evaluation of each proteolytic enzyme inhibitor of skin is beyond the scope of the present study since each enzyme may have innumerous inhibitors. Rawlings and Voegeli (2013) and Meyer-Hoffert (2009) reviewed the skin's

endogenous (and some exogenous) protease inhibitors. Both studies would assist in understanding the possible influence of inhibitors when enzymes were applied to the skin to act as exfoliating agents and assist in the choice of formulation components for enzymatic exfoliation.

2.2.2.3.2 Temperature

It is well established that the activity of enzymes is dependent on the temperature of the reaction. The enzymes present a temperature range that presents activity and a temperature that provides the highest activity, named optimum temperature. These parameters are specific for each enzyme, and it is correlated to the energy requirements to perform the reactions (WORTHINGTON; WORTHINGTON, 2016).

At non-extreme and healthy conditions, the skin surface temperature varies from 32 to 35 °C (LAI; ZHOU; CHEN, 2017). Lai, Zhou, and Chen (2017) demonstrated that uncovered regions, principally the face, have a higher temperature decrease when placed in a cold environment. In their studies, the skin temperature reached 19 °C after 45 minutes of exposure to an environment below 9 °C. The choice of proteolytic enzymes that present activity between 20 – 35 °C is recommended to avoid activity loss during the application. The use of warm water (LEES, 2012) (or other products) before applying the enzyme can improve the enzymatic exfoliation, enabling the skin temperature to match the temperature that the enzyme needs.

2.2.2.3.3 pH

Similarly to temperature, the enzymes present a pH range where they present catalytic activity (WORTHINGTON; WORTHINGTON, 2016). The skin is known to have an acid characteristic. Several factors can influence the pH of skin as body region, gender, lifestyle, and health, for example. The mean pH value of the superficies of the skin is close to 4.7 - 5.0 (LAMBERS et al., 2006; SEGGER et al., 2007). In his studies, Zlotogorski (1987) measured the pH values of the forehead and cheeks, obtaining, respectively, 4.0 – 5.5 and 4.2 – 5.9. Within this circumstance, it is important to highlight that the pH of the basal layer of the skin is approximate 7.4 (SMITH; BISHOP; NORTON, 2009), which can affect deeper exfoliation procedures.

The pH of the skin can be modified using external agents without damaging the tissue with water, cosmetics, and detergents/soaps (KORTING; BRAUN-FALCO, 1996). Blaak and Staib (2018) reviewed the relation of surfactants and the pH of the skin. The authors highlighted the pH shift values and pH recovery time after contact with surfactants on the skin. The simple application of water to wash or rinse the skin can modify the pH up to +1.0; on the other hand, the use of alkaline soap can cause the augment up to +3.0. The recovery time of pH after the treatment seems dependent on which product was applied and the shift that occurred, varying from 45 min to 8 or 12 h.

Regarding using enzymes as exfoliants, Smith, Bishop, and Norton (2009) suggested using proteolytic enzymes with sufficient activity at pH of 5.0 to 5.5 to be used as exfoliating agents. Papain (4.0 – 9.0), bromelain (5.0 – 8.5), ficin (5.0 – 9.0) (MAZORRA-MANZANO; MORENO-HERNÁNDEZ; RAMÍREZ-SUAREZ, 2018), pepsin (1.0 - 5.0), bacterial neutral proteases (5.0 – 8.0) (RANI, KIRTI; RANA, RACHITA; DATT, SANCHI, 2012), and keratinases (mostly alkaline, but there is some that act at 5.0) (GUPTA; RAMNANI, 2006) are some examples of proteolytic enzymes that have activity in the desired pH range. Some agents (water, cosmetics, toiletries, etc.) can be used before the enzyme application to reach the required pH value. The presence of water is important since the exfoliation is mainly a hydrolysis reaction, which requires water molecules. It is important to mention that, depending on the agent used, the structure of the proteolytic enzymes can be affected, so the combination of both agents must be used with care.

2.2.2.3.4 Enzyme stability in cosmetic formulations

The enzyme instability on cosmetic formulations is a major concern related to biological catalyzers as active ingredients. Generally, cosmetics contain various compounds in their formulation, affecting the enzyme catalytic activity or its structure, as oils and surfactants (SIM et al., 2000). For example, it is well known that surfactants can denature proteins, however, in the same way they are needed in cosmetic products. Moreover, the surfactants may bind with the active site of the enzyme by hydrophobic or electrostatic interactions. Nonionic surfactants should be preferred when enzymes are present since they provide lower activity loss than anionic and cationic amphiphiles (HOLMBERG, 2018). The correct choice of the formulation is needed to ensure that the

enzyme presents proteolytic activity, which cannot always occur in exfoliation products that contain enzymes (STREMNITZER et al., 2015).

Some studies have already studied the effects of stabilizers with proteolytic enzymes. Traversa, Machado-Santelli, and Velasco (2007) compared a cream and a gel formula containing papain for cosmetic application. The cream formula provided better enzymatic activity, even containing surfactant (nonionic) than the gel compost. The authors concluded that the increase in papain activity probably occurred due to the increased penetration and solubilization of lipids provided by the cream formula. Pinto et al. (2011) evaluated (by accelerated and normal stability tests) different formulas of emulsions containing papain in their composition. The authors demonstrated that the use of polyethylene glycol provided better results on activity. Sim et al. (2000) and Hara et al. (2014) provided better results when the authors stabilized papain in glucan and dextran using N, N-dimethylated casein, and keratin as substrates, respectively.

Another way to stabilize enzymes is using immobilization techniques. Enzyme immobilization comprehends the methods that can be used to immobilize the biological catalyzers on a support material. Different techniques can be used to immobilize an enzyme as following: adsorption; covalent attachment/cross-linking; and encapsulation/entrapment (MOHAMAD et al., 2015; REIS et al., 2019). Few studies have evaluated immobilization techniques for the enzymatic exfoliation application or similar procedures. For example, Chavan (2015) demonstrated that immobilized papain provided promising exfoliation results, stability, and reduced irritation. Other studies, as Manosroi et al. (2013) and Sahu, Kaurav, and Pandey (2017), showed that the encapsulation of papain improved stability, enhanced activity, and reduced skin irritation in wound management. Moreover, immobilization may also limit or reduce the digestion that the own proteases promote in themselves (CHAVAN, 2015).

2.2.3 Beauty face masks

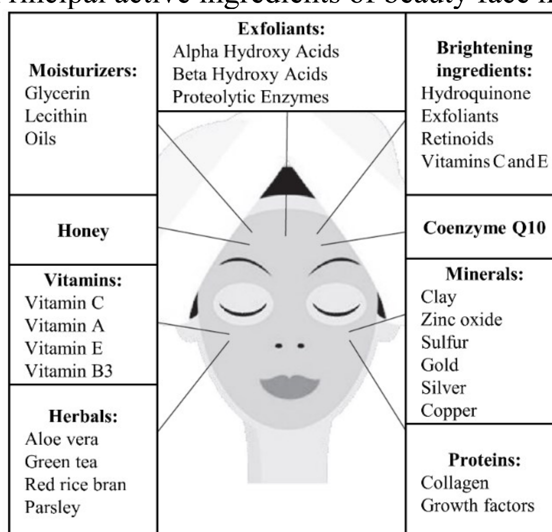
Facial masks are cosmetics products that were used to improve the visual of the skin since antiquity (BERINGHS et al., 2013). Despite the old reports of use, for a long time, the use of face masks was little exploited in the cosmetic sector. Due to social media and Asian skincare habits, the use of facial masks was boosted in the last years which turned facial masks into common and desired cosmetic products (MANSUY, 2017). Additionally, other factors improved the sales of beauty face masks: this type of product

is ready and easy to use and can be applied with other cosmetics without the need to stop other activities while using this type of product (MONTEIRO-RIVIERE, 2005). All these factors have helped in the rise of the face mask market (MANSUY, 2017), making them one of the most popular cosmetic products (CHU; WANG, 2018).

It is important to highlight that the face masks described in this section are related to beauty masks for cosmetic purposes. The term "face mask" can englobe masks to prevent diseases and infections (MORISHIMA et al., 2014), for the treatment of burns (COLLA et al., 2019; WEI et al., 2017), and to assist the facial reconstruction (KANT et al., 2017).

Traditionally, face masks do not contain many ingredients and have low unit prices. However, active ingredients can be incorporated into the face masks to bring new functionalities and greater and faster effectiveness. The main objective of the face masks is to promote fast and deep moisturization of the skin, eliminate oiliness and impurities, (MONTEIRO-RIVIERE, 2005) and act as a support to the release of the active ingredients. The main additives that can be incorporated into the masks are moisturizers, extracts or herbal ingredients, honey, proteins and growth factors, minerals, vitamins, enzymes and exfoliating agents, giving their respective effects to the masks (NILFOROUSHZADEH et al., 2018). Figure 2.4 shows some examples of active ingredients that were already incorporated into facial masks.

Figure 2.4: Principal active ingredients of beauty face masks.

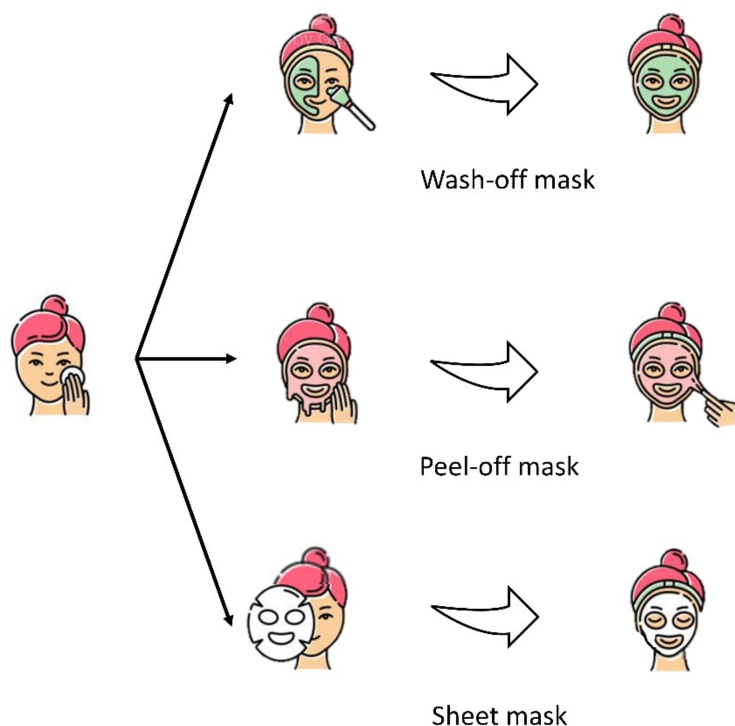


Source: adapted from Nilforoushzadeh *et al.* (2018).

The face masks can be classified as sheet, peel-off, and rinse-off (or wash-off) depending on the shape and method of application of the mask (MONTEIRO-RIVIERE,

2005; NILFOROUSHZADEH et al., 2018). Figure 2.5 relates and summarizes the main types of face masks and their respective components.

Figure 2.5:: Types of beauty face masks.



Source: author with imagens from iStock.

Sheet masks are thin materials that were applied to the skin and can mold its shape. This type of mask can be produced with paper, fabrics, non-woven, adhesive, hydrogels, and polymeric films (MONTEIRO-RIVIERE, 2005; NILFOROUSHZADEH et al., 2018). About 70% of all masks produced on the market are sheet type and are produced by non-woven fabrics consolidated by hydroentanglement (ZHAO et al., 2019). The main characteristics of sheet masks are enough flexibility to mold the face curvature and hydrophilicity (COLTELLI et al., 2018), good water retention, non-toxicity, enough water vapor transmission rate, and mechanical properties (AFONSO et al., 2019; ZHAO et al., 2019).

Different from sheet masks, peel-off masks are not solid materials. This type of mask is applied to the skin as a viscous gel, cream, or thick emulsions with enough rheological properties to be distributed homogeneity on the skin. This type of mask is named peel because contains film-forming agents and solvents that, when applied to the face, evaporate and form a thin layer on the skin (MONTEIRO-RIVIERE, 2005). The solvent more used is ethanol due to fast evaporation and better biocompatibility than other

solvents. In relation to film-forming agents, polyvinyl alcohol, polyvinyl acetate, and chitosan are some polymers that were used (NILFOROUSHZADEH et al., 2018).

Wash-off masks are named this way because they need to be washed off the face after their use. This type of mask has composition similarities with the peel-off masks but without the film-forming component. Mud, waxes, herbal formulations, propylene glycol, and petroleum derivatives were generally used to produce these masks (NILFOROUSHZADEH et al., 2018). Clay wash-off masks that are designed to remove impurities from the depths of the skin pores, provide essential minerals and nutrients for this organ, and provide refreshing effects, being the most suitable for those with oily skin (PAPINEAU, 2018).

Despite of the beauty face mask sector being growing in the past years, only few scientific studies that researched this type of cosmetics. This information can be observed in Table 2.3, which summarized the studies that evaluated facial masks between 2009 and 2021 for peel-off and wash-off masks. On the other hand, in Table 2.4 is showed the studies that researched sheet face masks for cosmetic application.

Table 2.3: Studies that produced and evaluated peel- and wash-off beauty face masks.

Type of mask*	Matrix	Active ingredient**	Reference
Peel-off	Polyvinyl alcohol	Rutin	(NISHIKAWA et al., 2007)
		Soy fermented	(VIEIRA et al., 2009)
		Green Clay and Aloe Vera	(BERINGHS et al., 2013)
		Soy fermented	(VELASCO et al., 2014)
		Silver nanoparticles	(BADNORE et al., 2019)
		Aloe Vera and monmorillonite	(ASTHANA et al., 2021)
	Polyvinyl alcohol, gelatin and hydroxypropyl methylcellulose	Red rice bran extract	(SUHERY; ANGGRAINI, 2016)
Wash-off	-	Yogurt and *Opuntia humifusa* powder	(YEOM et al., 2011)
		Dillenia indica pulp, licorice extract, honey	(SUTTHIPARINYANONT et al., 2013)
		Clay	(VELASCO et al., 2016)
		Clay and turmeric extract	(PAN-ON et al., 2018)

*: Not all studies identified the type of mask; **: Some components of the mask were not cited; -: none.

Table 2.4: Studies that produced and evaluated sheet beauty face masks.

Type of mask*	Matrix	Active ingredient**	Reference
Sheet	Bacterial cellulose	-	(AMNUAIKIT et al., 2011)
		Silk sericin	(ARAMWIT; BANG, 2014)
		Vitamin E	(REVENY; TANUWIJAYA; STANLEY, 2017)
		Diverse plant extracts	(PACHECO et al., 2018)
		Diverse extracts	(PERUGINI et al., 2019)
	PVA and collagen	Gold particles, vitamins A and E	(FATHI-AZARBAYJANI et al., 2010)
	Silk	-	(KOBAYASHI; SUMI, 2015)
	Hanji paper Non-woven fabric (cotton, cellulose and PET)	-	(KIM; PARK, 2016)
	Chitosan and Hyaluronic Acid	-	(LIBIO et al., 2016)
	Chitin and lignin Alginate and xanthan gum	- *Morinda citrifolia* extract	(MORGANTI et al., 2016) (SURINI; AULIYYA, 2017)
	PHA and starch Chitosan and cellulose	- -	(COLTELLI et al., 2018) (HE et al., 2018)
	Chitosan	Vitamin C and annatto powder	(AFONSO et al., 2019)
	Polyester elastomer fiber and viscose	-	(ZHAO et al., 2019)

*: Not all studies identified the type of mask; **: Some components of the mask were not cited; -: none; PET: polyethylene terephthalate; PHA: polyhydroxyalkanoate; PVA: polyvinyl alcohol.

2.2.3.1 Starch- and carboxymethyl cellulose-based films

Polysaccharide-based films are interesting materials to be used as sheet masks. They match some needed properties of this type of cosmetic such as hydrophilicity, flexibility, biocompatibility, and non-irritability (COLTELLI et al., 2018).

Starch is a semicrystalline polymer of plants. The main sources of starch are corn, cassava, potato, wheat, and sweet potato but this carbohydrate reserve can be found in other structures such as leaves, flowers, seeds, stems, roots, and fruits. Independently of the source, the starch granules present polymeric structures based on d- glucopyranose. The glucose units of this polysaccharide have α-1,4 and α-1,6 glycosides links which form

different polymers: amylose and amylopectin. Amylose is formed basically of linear chains of α-1,4 glycosides links (and 2 – 5% of α-1,6 glycosides links). On the other hand, amylopectin presents both glycoside links with branches. Amylopectin linear structure has, on average, 20 to 25 anhydroglucose residues linked by α-1,4 bonds; the branches are linked by α-1,6 glycosidic bonds and connect the linear structures (RATNAYAKE; JACKSON, 2008; TAGHIZADEH; SABOURI, 2013).

The semicrystalline structure of starch granules is reduced or destroyed during heating. This process is named gelatinization and comprehends the lixiviation of both amylose and amylopectin from the granules (SOUZA; ANDRADE, 2000). The temperature of gelatinization varies from the source of the starch and is nearly 80 °C. The gelatinization is useful because allows the processability of starch-based materials (COLTELLI et al., 2018).

Starch is known to be the most important polysaccharide used to produce biodegradable films due to its abundant and renewable source and to form continuous matrices. However, starch present high hydrophilicity and poor mechanical properties when compared to other polymers (GHANBARZADEH; ALMASI; ENTEZAMI, 2010). One method to minimize or overcome the undesired properties of a polymeric material is blending two or more polymers. Diverse polymers can be blended with starch to produce films, but blending starch with another polysaccharide is interesting to allow the production of homogeneous and continuous matrices. The starch and carboxymethyl cellulose (CMC), a cellulose derivative, has already been shown to produce films with interesting properties (CHANG; YU; MA, 2009).

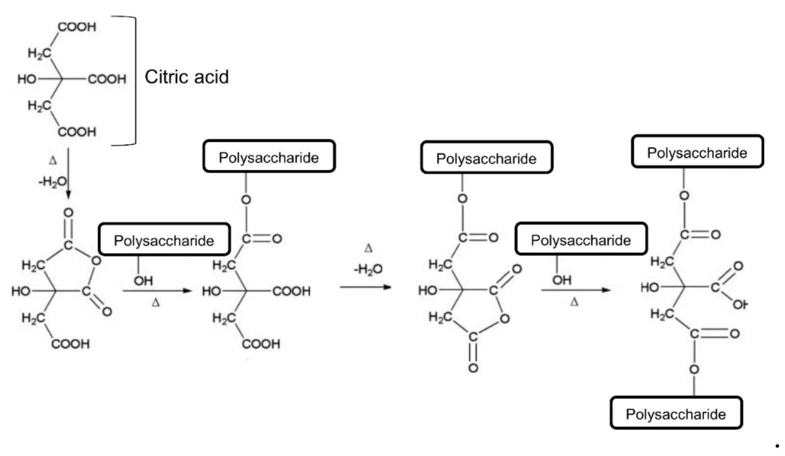
CMC is a linear polysaccharide that is obtained by the substitution of part of the hydroxyl groups by carboxymethyl groups of the glucose unit. Sodium CMC is a copolymer formed by two units: β-D-glucose and β-D-glucopyranose 2-O-(carboxymethyl)-monosodium salt which is linked via β-1-4-glycosidic bonds (CHARPENTIER et al., 1997). The polymeric matrices produced with CMC are known to present wide applicability and low cost. Generally, films of CMC are flexible, transparent, easy to process, non-allergenic or non-toxic (BIFANI et al., 2007; HASHEMINYA et al., 2018).

Films of starch and CMC have high hydrophilicity and water holding capacity, flexibility to mold the skin, low toxicity, and biodegradability (CHANG; YU; MA, 2009). However, the blended matrices still tend to tack tensile strength and water resistance, properties which may minimize the possible use of the desired material. Generally,

crosslinking agents are used to produce more cohesive matrices and overcomer these undesired properties. Citric acid is a crosslinker of polysaccharides and other biopolymers that promotes covalent bonds and additional hydrogen bonds due to its multi-carboxyl structure (GHANBARZADEH; ALMASI; ENTEZAMI, 2010). The employ of citric acid as a crosslinker of polysaccharide materials occurs due to its biocompatibility, water solubility, non-toxicity, and low cost (URANGA et al., 2020).

Figure 2.6 details the mechanism of crosslinking of polysaccharides with citric acid. When heated, citric acid is dehydrated and forms a cyclic anhydride structure. This structure reacts successively by esterification with the carbohydrate molecule. New anhydride structures were obtained which allow the bond with other hydroxylic groups of the polysaccharide that also esterify with other hydroxyl groups and form the crosslinks (DEMITRI et al., 2008; XIE; LIU; CUI, 2006). The presence of carboxylic acid and hydroxyl groups in the crosslinks reduces the hydrophilicity and improves the cohesion of the polymeric matrix. There is, also, the provision of additional hydrogen bonds and binding sites that can be used to incorporate molecules of interest (GHORPADE; YADAV; DIAS, 2016).

Figure 2.6: Possible crosslinking mechanism of citric acid in polyssacharides.



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Source: adapted from Demitri *et al.* (2008).

Moreover, this blend can be crosslinked with citric acid which may reduce the Despite of several studies that have evaluated the production of films of the blend of starch and CMC (GHANBARZADEH; ALMASI; ENTEZAMI, 2010; GHANBARZADEH; ALMASI; OLEYAEI, 2013; PUTRI; SETIAWAN; ANGGRAINI, 2017; SURIYATEM; AURAS; RACHTANAPUN, 2019; TAVARES et al., 2019;

TONGDEESOONTORN et al., 2011), no previous study had evaluated this type of material to act as a beauty mask.

2.3 PAPAIN

Papain (EC 3.4.22.2) is a cysteine protease of plant origin that is isolated from papaya latex (*Carica papaya* L.). This enzyme presents only one polypeptide chain of 212 amino acids and has 23.4 kDa of molar mass, approximately. The domains of papain contain three disulfide bonds (Cys56–Cys95, Cys22–Cys63, and Cys153-Cys200) and one sulfhydryl group in the active site of the enzyme at residue Cys25 (LACHMANOVÁ et al., 2016). Papain has a wide range for substrate specificity acting, preferably, in arginin and lysin or phenylalanine residues that were followed by other amino acids (AMRI; MAMBOYA, 2012). Besides proteolytic activity, papain also exhibits endopeptidases, aminopeptidases, dipeptidyl peptidases, esterase, amidase, transamidase, thiolesterase, and transesterase activities (MESHRAM et al., 2019).

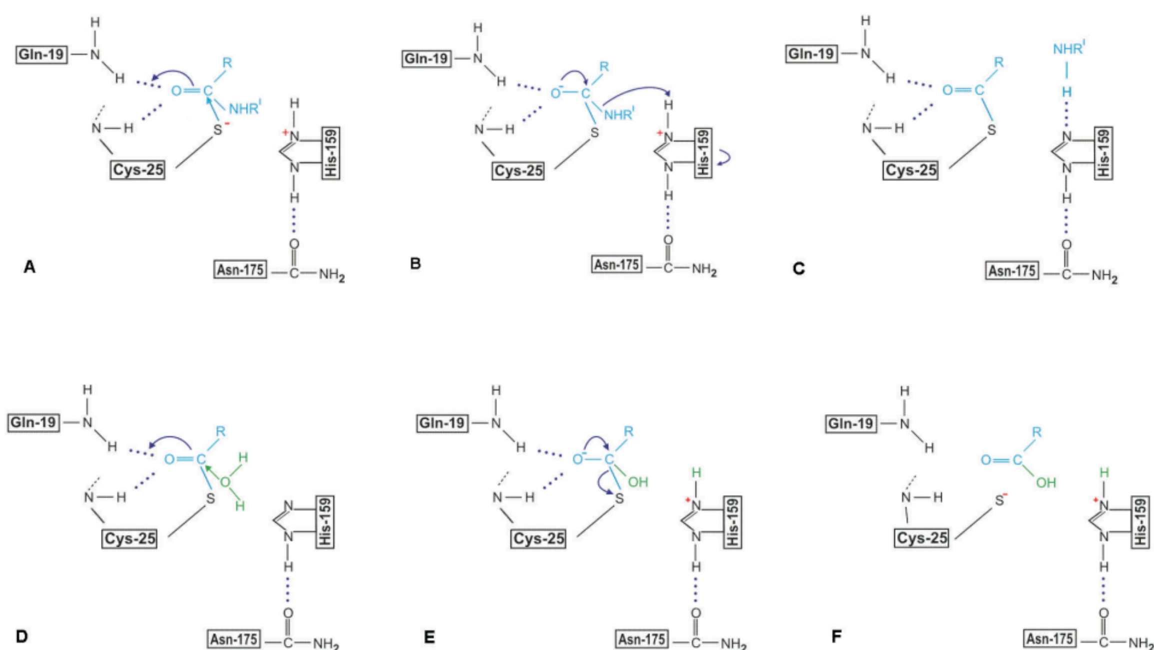
Cysteine proteases have three principal residues in the active domain: Cys25, His159, and Asn175. After the bonding of the peptide chain in the active site of the enzyme, the mechanism of the action of papain starts with the deprotonation of Cys25 residue by action of His159 residue. This result in a highly nucleophilic thiolate/imidazolium ion pair which allows the Cys25 to perform a nucleophilic attack in the carbonyl group of the peptide backbone (AMRI; MAMBOYA, 2012).

A first thioester tetrahedral intermediate is produced after the release of the amino or amine terminal fragments of the substrate. This intermediate is stabilized by hydrogen bonds between the oxyanion of the substrate and the Gln19 residues of the enzyme (Figure 2.7A-B). The amine substrate bonds His159 whereas the carboxylic part is the Cys25 residue (Figure 2.7C). Then, the aminic part is dissociated and replaced by water molecules (Figure 2.7D). Finally, a second tetrahedral intermediate is formed by hydrolyzing the thioester bond and, consequently, producing a carboxylic acid fragment of the remaining substrate. The enzyme is regenerated (Figure 2.7E-F). Differently of the other residues, Asn175 residue did not actively participate in the reaction but only assist in the orientation of the imidazole ring of His159 residue (AMRI; MAMBOYA, 2012; RZYCHON; CHMIEL; STEC-NIEMCZYK, 2004; VERMA; DIXIT; PANDEY, 2016).

Papain is the most commercialized enzyme in the protease market, outselling other plant-based and microbial proteases (FERNÁNDEZ-LUCAS; CASTAÑEDA;

HORMIGO, 2017). Different industrial sectors have been using papain. Beyond its classic use in the beverage (CERRETI et al., 2017; GOMAA, 2018) and meat industries (ISTRATI, 2008; JUN-HUI et al., 2020; SAEED et al., 2017), products and processes with papain appeared in pharmaceutical, biomedical and cosmetical fields. Papain can act to debride wounds (LANGER et al., 2013; SINGH; SINGH, 2012), depilate (TRAVERSA; MACHADO-SANTELLI; VELASCO, 2007), assist the removal of scars (MANOSROI et al., 2013), enhance the transdermal release of drugs (PINTO et al., 2007; SIM et al., 2003), exfoliate (as earlier described in section 2.2.2), etc.

Figure 2. 7: Catalytic mechanism of cysteine proteases.



Source: modified from Rzychon, Chmiel, and Stec-Niemczyk (2004).

It is important to highlight that the use of different formulations and matrices has been studied to increase the stability of papain (HARA et al., 2014; PINTO et al., 2007, 2011; SIM et al., 2000; TRAVERSA; MACHADO-SANTELLI; VELASCO, 2007). A general approach to attempt to increase the stability of enzymes occurs by immobilizing the catalysts in different matrices.

2.3.1 Immobilization of papain in polymeric films

There are two main aims when immobilizing an enzyme. The first is related to reducing the instability that is caused by temperature, pH, light, oxygen, and general storage conditions which affect the catalytic activity of enzymes. The second reason is to reduce economic and environmental problems since the support may assist the enzyme recuperation and reuse (IMAM; MARR; MARR, 2021). Traditionally, the immobilization techniques can be divided into four different groups: entrapment, adsorption, crosslinking, and covalent bonding. The first two methods occur with physical bonding between enzyme and support and the last two are more related to chemical bonding and, generally, use specific agents to promote this type of immobilization (CEN et al., 2019).

When an enzyme is immobilized by entrapment, the catalyst is trapped within a solid or semisolid material. This technique is based on the physical impediment of the release of the enzyme from within the support. On other hand, the support must allow the substrate and product mass transfer. Depending the material, additional covalent bonding can be used to improve the maintenance of the enzyme inside the support. Entrapment is a simple technique to irreversibly immobilize enzymes. The limitations of the technique are related to mass transfer problems and enzyme loss, for example (MOHAMAD et al., 2015; SHELDON, 2007).

Adsorption is a physical method in which each of the enzymes can be adsorbed by ionic, entropic, hydrophobic, van der Waals, affinity, etc. mechanisms in the support. Ionic strength and pH of the protein solution and the morphology and chemical composition of the support affect this type of immobilization (SAHIN; OZMEN; KIR, 2015). Adsorption is a method that is considered reversible and easy to do be performed for most enzymes. However, generally, the physical bonds obtained by adsorption are weaker than the ones produced by covalent bonding, which can limit the use of this technique (DATTA; CHRISTENA; RAJARAM, 2013).

In the crosslinking method, a material crosslinks enzymes with each other, with another protein, and/or with support material. Generally, this technique is performed by using a homo- or heterofunctional agent. Depending on the support, both ionic and covalent crosslinks can be used to immobilize enzymes. Among the crosslinking agents, glutaraldehyde, benzoquinone, dextran polyaldehyde, polyethyleneimine, and carboxylate activating carbodiimide are the most used. When the enzyme is crosslinked

without support this result in crosslinked enzyme aggregates (CLEA) or crosslinked enzyme crystals (CLEC) (IMAM; MARR; MARR, 2021; REIS et al., 2019).

The last type of enzyme immobilization happens when the enzyme is bonded to the support by covalent bonding. The functional and active site amino acid residues are avoided to prevent enzyme activity loss in the covalent bonding, demonstrating that the direction of the immobilization is crucial. There is an optimum enzyme orientation (MOHAMAD et al., 2015). The most common residues that were used to immobilize were arginine and lysin, for amine ($–NH_2$), glutamic or aspartic acid, for carboxylic groups (–COOH), threonine and serin, for hydroxyl groups (–OH), and cysteine, for thiol groups (–SH). This type of immobilization tends to present high resistance to detaching, being considered an irreversible method (HASSAN; TAMER; OMER, 2016). Single or multiple bonds can be used to immobilize (REIS et al., 2019).

In covalent bonding, the support can have the desired reactive groups to attach the enzyme and/or be chemically modified to do that (HASSAN; TAMER; OMER, 2016). Conceptually, functionalization or derivatization is a procedure whereby a new chemical function is introduced on support. The next step is named activation. In activation, the chemical function groups that were achieved in the functionalization were made reactive for the enzyme. Both processes can be performed at the same time (ZUCCA; SANJUST, 2014).

There are diverse agents that can be used to functionalize and/or activate the support. The choice of a specific compound depends on the enzyme, the support material, and the desired application. Chemical agents such as glutaraldehyde, 1-ethyl-3-(3-dimethylaminopropyl) (EDC) with or without the use of N-hydroxysuccinimide (NHS) or ethylenediamine (EDA), and periodate oxidation have been used (ZUCCA; SANJUST, 2014). Moreover, the application of technologies such as ultraviolet radiation induction, gamma irradiation, or plasma application can also provide covalent bonding (CEN et al., 2019).

As already mentioned, papain has a prominent position in the industry and is applied in different sectors. Despite its wide applicability in its native form, the immobilization of papain can provide advantages and even more uses. Diverse supports have been used to immobilize this enzyme, with both organic and inorganic materials: agarose, chitosan, alginate, cotton, silicates, inorganic oxides, magnetic particles, polymeric membranes, etc. (TACIAS-PASCACIO et al., 2021). In the case of cosmetic application of papain, semisolids, as creams and gels, or solids formulations (PINTO et

al., 2011), as polymeric matrices, can be used. The stabilization by immobilizing the enzyme can also prevent possible irritation effects that direct contact with the enzyme and skin could promote (HARA et al., 2014).

Polymeric films (or membranes) are one of the most versatile supports used to immobilize enzymes. Depending on the use or desired characteristics, synthetic and bio-derived polymers can be used (DATTA; CHRISTENA; RAJARAM, 2013). Generally, these materials present a high surface area and their properties can be easily modified (ZDARTA et al., 2018). Table 2.5 summarizes the last studies that immobilized papain in this type of support. In general, two interesting results can be highlighted. Most studies that involved immobilizing papain in films aimed the application as wound dressing or food packaging materials. From the methods. Approximately, half of the works used physical methods to immobilize papain; from the chemical immobilization, the studies which used glutaraldehyde were more evident than other derivatization and/or activation molecules.

Table 2.5: Immobilization of papain in polymeric films and membranes and their recovered activity (RA) and immobilization yield (IY).

Material	Immobilization method	Application	Result*	Reference
Starch film	Entrapment	Food packaging	Not described	(WONGPHAN et al., 2022)
Bacterial cellulose membrane	Adsorption and glutaraldehyde activation	Wound dressing	Not described	(ASANARONG et al., 2021)
Chitosan film	Entrapment	Food packaging	Not described	(SANTOS et al., 2021a, 2021b)
Bacterial cellulose membrane	Adsorption (pH 7 at 45 °C for 24 h)	Wound dressing	RA: 93% IY: 53%	(VASCONCELOS et al., 2020a, 2020b)
Chitosan-montmorillonite composite film	Crosslinking with glutaraldehyde	Wine clarification	IY: 60%	(BENUCCI et al., 2020)
Alginate film	Adsorption (25 °C, pH 5.8)	Wound dressing	RA: 80%	(MOREIRA FILHO et al., 2020)
Deacetylated cellulose membrane	Covalent bond with aldehyde (AL) or glyoxylic (GL) activated groups	Membrane chromatography	IY: 38% (AL) IY: 63% (GL)	(DEL MONTE-MARTÍNEZ et al., 2017)
PVA/alginate film	Entrapment	Wound dressing	Not described	(DUTRA et al., 2017)
PVA/cashew gum film	Covalent bonding (metaperiodate)	Wound dressing	RA: 97%	(SILVA et al., 2016)
PVA electrospun membrane	Crosslinking with glutaraldehyde vapor	Biosensor	IY: 33%	(MORENO-CORTEZ et al., 2015)
Polyethylene (PE) film Polyprolactone (PCL) film	Crosslinking in curcumin activated with ultraviolet radiation	Food packaging	IY: 92% (PE) IY: 90% (PCL)	(MANOHAR et al., 2015)
Polyurethane film	Crosslinking with glutaraldehyde	Food packaging	RA: 88%	(MANOHAR et al. 2014)
PVA electrospun membrane	Entrapment	Wound dressing	IY: 50%	(SHOBA et al., 2014)
Chitin film	Entrapment	Wound dressing	Not described	(SINGH; SINGH, 2012)

*: Values of RA and YI are the highest that are described in each study; PVA: polyvinyl alcohol;

2.4 CONSIDERATIONS ON LITERATURE REVIEW

Papain and other proteolytic enzymes can promote the hydrolysis reaction of the proteins of the skin and, consequently, act as exfoliating agents of this organ. Some factors that affect enzyme catalysis as temperature, pH, presence of activators and/or inhibitors, enzyme concentration, etc., and must be considered when using enzymes as active ingredients in cosmetic formulations. Despite the commercial presence of exfoliating products containing enzymes as an active ingredient, the evaluation of this type of application has been neglected in scientific studies in recent years.

There are diverse formulations that can be produced to apply an exfoliant to the skin. Some studies have demonstrated that the use of stabilizing agents can help the enzyme action by allowing it reactional control. In general, immobilizing enzymes on support tends to improve the stability of the biocatalysts. Aiming the cosmetic application, papain can be immobilized in face masks to give the exfoliating activity to this cosmetic.

Beauty face masks are used to improve the skin appearance by prolonging the action of other cosmetics or due to containing active ingredients. There are three types of masks: sheet, wash-off, and peel-off. In terms of composition, several matrices and other ingredients can be used to produce them. In recent years, industries and consumers have preferred to use biodegradable materials to avoid environmental problems. Starch- and CMC-based polymeric films present interesting properties to be used as face masks.

This study is structured in the theory that papain can promote the hydrolysis of proteins of the skin and that the immobilization of this enzyme in starch- and CMC-based polymeric may produce a face mask with interesting properties that present exfoliating activity. This is an unprecedented and promising application, contributing to the advance in the application of biocatalysis in the cosmetology area.

CHAPTER 3: *IN VITRO* EFFECT ON THE PROTEOLYTIC ACTIVITY OF PAPAIN WITH PROTEINS OF THE SKIN AS SUBSTRATE

This section is based in related to the first experimental article. This article was published in International Journal of Cosmetic Science as "*In Vitro* Effect on the Proteolytic Activity of Papain with Proteins of the Skin as Substrate". This article was accepted in 2022 and indexed (https://doi.org/10.1111/ics.12805). According to Wiley subscription rules, the authors retain the right to include the article in a thesis, provided it is not published commercially.

3.1 INTRODUCTION

Papain (EC 3.4.22.2) is a proteolytic enzyme extracted from the latex of papaya (*Carica papaya*)) (AMRI; MAMBOYA, 2012). This enzyme has a wide range of substrate specificity, preferably acting on peptide bonds involving arginine and lysine or phenylalanine, leucine, and glycine residues residues (AMRI; MAMBOYA, 2012; PINTO et al., 2011). Papain is the most commercialized enzyme in the protease market, outselling other plant-based and microbial proteases (FERNÁNDEZ-LUCAS; CASTAÑEDA; HORMIGO, 2017). Generally, papain is used with thiol activator agents, mainly cysteine molecules, since these reducing agents may improve the rate of substrate-enzyme decomposition, and consequently, the reaction rate (HOMAEI et al., 2010).

Plant proteases present various interesting physiological properties that are needed for the maintenance of the plant and that can be extrapolated to the industrial use of this type of enzyme (HOMAEI et al., 2017). In addition to the classic use of papain in the food and beverage industry, other segments also use the enzyme in their processes and products due to the broad substrate specificity and range of operational stability (pH and temperature) (FERNÁNDEZ-LUCAS; CASTAÑEDA; HORMIGO, 2017). The cosmetic, medical, and pharmaceutical segments have started to use papain to improve product quality due to the increase in the use of bio-derived and eco-friendly ingredients (SHOUKET et al., 2020; SUNAR; KUMAR; DESHMUKH, 2016). Papain has been applied as a debridement agent of wounds (LANGER et al., 2013; PORSANI et al., 2016; SINGH; SINGH, 2012), exfoliation agent (BANCHHOR; SARAF, 2008; TREVISOL et al., 2021), depilatory agent (TRAVERSA; MACHADO-SANTELLI; VELASCO, 2007), and transdermal drug permeation enhancer (SIM et al., 2003), for example. The chemical

stability of papain, which may decrease depending on the storage and presence of other ingredients in the formulation, is the most evaluated effect in terms of skin application, principally related to the complexation of the enzyme with polymers (HARA et al., 2014; PINTO et al., 2007, 2011; SIM et al., 2000; TRAVERSA; MACHADO-SANTELLI; VELASCO, 2007).

Each dermic application of papain has its specificity; however, all of them are related to the capability of the enzyme to act on the human skin, promoting the hydrolysis of proteins and their structures (HARA et al., 2014; LANGER et al., 2013; PINTO et al., 2011). The proteins that compose the skin are keratin, collagen, elastin, and some proteoglycans (HIRAO, 2017). Due to its structure, keratin presents resistance to hydrolysis against common proteolytic enzymes (NAVONE; SPEIGHT, 2018). Even so, some studies have already demonstrated that papain presents keratinolytic activity (HARA et al., 2014; JIN et al., 2017; RAMNANI; GUPTA, 2007). Similarly, Lukin (LUKIN, 2020), Sorapukdee et al. (SORAPUKDEE et al., 2020), and Tan et al. (TAN; CHANG; MENG, 2019), for example, have demonstrated the collagenolytic activity of papain.

An interesting aspect to be evaluated in the topical and transdermal formulation of cosmetics and pharmaceuticals is the concentration of the active ingredient. This aspect is increasingly important depending on the costs of the active ingredient (TAN; CHANG; MENG, 2019). Moreover, enzymes are pH, temperature, pressure, ionic strength, etc. dependent, acting over a particular range of physicochemical parameters (BAHRAMI; HOMAEI, 2018). All these parameters must be evaluated to obtain a formulation that presents the desired activity, a problem that occurs in some topical formulations (WIECHERS et al., 2004). Despite that are studies and products that use papain to be applied on the skin, to our knowledge, there is no data regarding the effects of papain concentration, contact time, and the influence of activators and reducing agents on the hydrolysis of proteins of the skin aiming this type of application. In this study, we evaluate how the enzyme concentration, pH and temperature (ranges of the human skin), and presence of the reducing agents cysteine and sodium sulfide affected enzyme activity focusing on the dermic application of the enzyme. Casein, collagen, and keratin were studied as substrates.

3.2 MATERIAL AND METHODS

3.2.1 Material

Papain from *Carica papaya* (activity ≥ 3.6 units mg^{-1} protein) was purchased from Sigma-Aldrich Chemical Co. (USA). Casein was obtained from Dinâmica Química (Brazil), turkey feathers (keratin source) were obtained from Plumas & Penas (Brazil), and collagen powder, extracted from bovine hides, was donated by Novaprom Food Ingredients Ltda. (Brazil). Tyrosine (Synth, Brazil) and leucine (VETEC, Brazil) were used to build the standard curves. L-Cysteine hydrochloride monohydrate, ethylenediaminetetraacetic acid (EDTA), ninhydrin reagent (Vetec, Brazil), polyethylene glycol (PEG) 3500, sodium sulfite (Synth, Brazil), and trichloroacetic acid (TCA, Neon, Brazil) were used in the measurement of the proteolytic activities. Skin sections of freshly slaughtered pigs (obtained from a local slaughterhouse) were cleaned with distilled water and stored in refrigeration before their uses. All reagents were of analytical grade quality.

3.2.2 Caseinolytic activity assay

The caseinolytic activity was measured according to previous studies (HOMAEI et al., 2010; RANJBARI et al., 2019; RAZZAGHI; HOMAEI; MOSADDEGH, 2018),, with some modifications. Briefly, in 2 mL microtubes (n = 4), 0.5 mL of papain solution (prepared in 100 mM sodium phosphate buffer, pH 7.5) was mixed with 0.5 mL of 0.65% (w v-1, pH adjusted to 7.5) casein solution and incubated for 5 min at 37 °C. Different final papain concentrations in the microtubes were evaluated: 0.1 to 2.0 mg mL^{-1}. Before use, both papain and casein solutions were maintained at 37 °C for 20 min. The reaction was stopped by adding 0.5 mL of 10% (w v^{-1}) TCA. Then, the microtubes were kept at room temperature for 30 min and centrifuged for 5 min at 8,170 xg. The control samples were performed as described, but the enzyme solution was added after the TCA solution. The absorbance of the supernatant was observed at 280 nm in UV/VIS spectrophotometer (U-2900, Hitachi, Japan) and correlated with a tyrosine standard curve. One unit of caseinolytic activity (PU) was defined as the amount of enzyme required to liberate 1 μmol of tyrosine per minute under the defined assay conditions.

3.2.2.1 Effects of cysteine concentration during the reaction

The effect of cysteine addition to the reaction medium was evaluated on caseinolytic activity. The concentration of cysteine varied from 0 to 48 mM. The amino acid was solubilized in the enzyme solution prior the mixture with the substrate.

3.2.3 Collagenolytic activity assay

The collagenolytic activity was measured according to Souchet and Laplante (2011), with modifications. Briefly, 1 mL of 100 mM sodium phosphate buffer (pH 7.5) was added to microtubes (2 mL) containing 10 mg of collagen powder. The suspension was incubated at 37 °C for 20 min. Then, 0.25 mL of papain solution (also prepared in sodium phosphate buffer) was added, and the mixture was incubated at 37 °C for 1 h. The papain final concentrations varied from 0.1 to 2.0 mg mL^{-1} and the concentration of cysteine was chosen according to the previous assays. After the incubation, the microtubes were maintained for 5 min in a cold bath and centrifuged for 5 min at 8,170 xg. The supernatant was recovered and used to determine the collagenolytic activity using the ninhydrin method. The control samples were performed similarly, with the addition of papain solution only after the removal of the non- solubilized collagen powder in the centrifugation step.

The ninhydrin reaction, adapted from Zhang et al. (2013), was used to quantify the amino acids that were released from the reaction. Firstly, a 1.6 mg mL^{-1} solution of $SnCl_2 \cdot 2H_2O$ was prepared in 100 mM sodium citrate buffer (pH 5.0), and N_2 was purged into the solution. Separately, ninhydrin was dissolved in dimethyl sulfoxide using a magnetic stirrer until the final concentration of 50 mg mL^{-1}. Then, both $SnCl_2 \cdot 2H_2O$ and ninhydrin solutions were mixed with equal volumes. The final solution was used to measure the collagenolytic activity.

For measurement, in 2 mL microtubes, 0.15 mL of the sample supernatant of the reaction of papain with the substrate was mixed with 0.15 mL of 25 mM of EDTA and 12% (w v^{-1}) PEG 3500 solution. Then, 0.75 mL of the $SnCl_2 \cdot 2H_2O$-ninhydrin solution was added and the microtubes were heated at 80 °C for 10 min (microtubes cap closed). The microtubes were cooled using cold water for 3 min, and 0.75 mL of deionized water was added. The amino acids were analyzed using a spectrophotometer (BEL SP1105, Brazil) at 570 nm and correlated to a leucine standard curve. One unit of collagenolytic

activity (CU) was defined as the amount of enzyme that released 1 μmol of leucine per hour under the experimental conditions. The assay was performed in quadruplicate.

3.2.4 Keratinolytic activity assay

The keratinolytic activity was determined by the method of Wu et al. (2017), with modifications. Firstly, the feathers were rinsed to remove the impurities and dried (at 40 °C) for 24 h. Manually, the shaft and rachis were discarded from the feathers. Then, the barbs and afterfeather were ground using a manual mincer until they passed through a 4-mm sieve. The obtained feather substrate was maintained at 4 °C before its use.

Feather substrate suspension was obtained by adding 4 mL of 100 mM sodium phosphate buffer solution (pH 7.5) to 25 mg of solids (previously sieved feather substrate) in centrifuge tubes of 15 mL (n = 4). The tubes were maintained at 37 °C for at least 20 min, and then 1 mL of papain solution was added; the final papain concentration varied from 0.1 to 2.0 mg mL^{-1}. The tubes were incubated at 37 °C for 1 h. After the incubation, the solution was vacuum filtered (Whatman No. 1 filter paper) to remove the feather substrate excess, and the filtered samples were centrifuged for 10 min at 1,780 xg. The supernatant amino acid concentration was determined by the ninhydrin method. The control samples were prepared similarly, with only the addition of papain solution occurring after the filtration step.

3.2.4.1 Effects of cysteine and sodium sulfite in the keratinolytic activity

Cysteine and sodium sulfite molecules can improve the hydrolysis of keratin, acting as reducing agents and facilitating the papain action. Sodium sulfite (0.2 – 0.4% (w v^{-1})) and cysteine (3 and 24 mM) were added before the papain solution, and their effects on the keratinolytic activity were analyzed.

3.2.5 Determination of degree of hydrolysis (DH)

The DH of casein, collagen, and keratin were evaluated by the ninhydrin method with some modifications. All the experiments were conducted similarly to the determination of proteolytic but with modification of the reaction time. Then, all the hydrolyzed samples were analyzed by the ninhydrin method. The DH was calculated by

Equation 3.1, where $NH_2(t)$ was the concentration of α-amino groups released in the mixture at time t by the enzyme; $NH_2(t_0)$ was the initial concentration of α-amino groups; and $NH_2(t_{total})$ was the concentration α-amino groups in the mixture determined after acid hydrolysis (HCl 6 M, 24 h at 110 °C) (REYNAUD et al., 2020).

$$DH\ (\%) = \frac{NH_2(t) - NH_2(t_0)}{NH_2(t_{total}) - NH_2(t_0)} \times 100 \qquad \text{[Eq. 3.1]}$$

3.2.6 Effects of temperature and pH in the enzyme proteolytic activities

The determination of PU, CU, and KU was also performed varying the temperature incubation or pH of the buffer solution used. The thermal influence on the proteolytic activities on casein, collagen, and keratin substrates was evaluated in 100 mM sodium phosphate buffer, pH 7.5, at 26, 30, 34, and 37 °C. Similarly, the pH stability was checked by incubating the samples at 37 °C and varying the pH by using 100 mM sodium acetate buffer at pH 4.5 and 5.5 and 100 mM sodium phosphate buffer at pH 6.5 and 7.5.

3.2.7 *In vitro* evaluation of the topical application of the enzyme on skin substrate

The porcine skin was rinsed to remove the impurities and the hair and fat excess were manually removed with help of scissors. The fragments were cut into pieces of 2 cm^2, approximately. Then, 75 µL of sodium phosphate buffer (100 mM, pH 7.5) was added over the epidermal layer of the skin to hydrate and maintain the pH of the region to the desired one. After 5 min, 75 µL of papain solution (0.5 and 1.0 mg mL^{-1}), also prepared in phosphate buffer (100 mM, pH 7.5), was added to the substrates. The pieces were washed by immersing into phosphate buffer after 30 min of incubation at 37 °C. Negative control was performed by only using the buffer solution and positive control when 75 µL of 10 % (w v^{-1}) TCA solution was used.

The skin fragments were immersed in 2.5 % (w v^{-1}) glutaraldehyde-phosphate buffer solution (50 mM, pH 7.4) for 2 h. After the fixation, the skin fragments were washed in phosphate buffer solution to remove the glutaraldehyde excess. The samples were pos-fixated by immersing them in a 1.0 % (w v^{-1}) osmium tetroxide-phosphate buffer solution for 2 h. The samples were washed with distilled water to ensure the removal of all unbound osmium tetroxide molecules. Then, the samples were dehydrated

by immersing them in increasing concentrations of ethanol and critical point dried (EM CPD 030, Leica, Germany). The dried fragments were placed on stubs and coated with an ultrathin gold layer and analyzed with scanning electron microscopy (model JSM-6390LV, JEOL, Japan) with an accelerating voltage of 10 kV.

3.2.8 Statistical Analysis

The responses of the assays were evaluated by analysis of variance (ANOVA) followed by the Tukey test, with a confidence interval of 95%. All the experiments were randomized.

3.3 RESULTS AND DISCUSSION

3.3.1 Effect of cysteine concentration on caseinolytic activity of papain

It is well established that the addition of reducing agents containing thiol groups such as cysteine, sulfite, sulfide, thioglycolate, and cyanide improves papain proteolytic activity. These molecules act as activators (ARNON, 1970) and as antioxidant (SRINIVASAN et al., 2017) agents for the papain action. Among them, cysteine has more highlights in the literature. Cysteine (and other sulfhydryl-reactive compounds) improves the proteolytic activity by maintaining the Cys-residue in the active form in the active site and, consequently, keeping the active conformational structure of the enzyme (BAHRAMI; HOMAEI, 2018). Several studies and protocols have already demonstrated and cited the importance of adding cysteine with papain. However, the amino acid concentration in each study is discrepant, varying from 0.05 to 50 mM (ARNON, 1970; HOMAEI et al., 2010; YAO et al., 2013).

As shown in Table 3.1, L-cysteine hydrochloride addition improved ($p > 0.05$) the caseinolytic activity of papain. However, no statistically significant improvement ($p < 0.05$) was observed when cysteine concentration varied from 3 to 48 mM. Sanner and Pihl (1963) demonstrated that cysteine act as an activator to papain at a concentration above 0.01 mM. Similarly, to present work, no improvement was observed when cysteine concentration increased from 1 to 10 mM (α-benzoyl-L-arginine amide was used as substrate). Bahrami and Homaei (2018) observed that the increase from 3.5 to 5 mM of cysteine did not improve the caseinolytic activity of *Penaeus vannamei* protease. On the

other hand, the increment of cysteine concentration improved the enzyme activity. The present studies can be explained by the fact that a lower concentration of cysteine was not tested, indicating that the 3 mM was sufficient to improve the papain activity.

In another study, Homaei et al. (2010) studied the presence of 0 – 40 mM of cysteine in the caseinolytic activity of papain. These authors observed that the use of 5 and 10 mM of the amino acid improved the enzyme activity with the highest activation occurring with 10 mM. The use of 20 mM or more of cysteine had no inhibition effects. When comparing the results of these authors with the present work the differences are not clear but may be related to the enzyme source, purity, reaction medium composition, and enzyme and substrate ratio.

Table 3.1: Effect of cysteine concentration on the caseinolytic activity of papain.

L-Cysteine hydrochloride (mM)	Caseinolytic Activity (PU mL^{-1})
0	0.54 ± 0.02 [b]
3	1.54 ± 0.09 [a]
6	1.52 ± 0.04 [a]
9	1.43 ± 0.06 [a]
12	1.52 ± 0.08 [a]
24	1.57 ± 0.04 [a]
48	1.59 ± 0.05 [a]

Assay's condition: 37 °C, pH 7.5, and 0.5 mg mL^{-1} of papain.

Average ± standard deviation of experimental determinations. Averages with the same letter, in the same column, indicate no significant differences ($p < 0.05$) by the Tukey test.

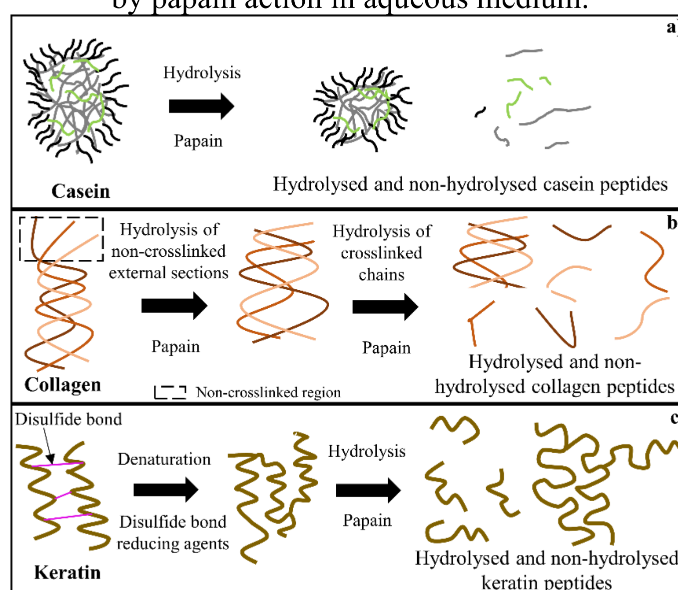
Papain can also be reversibly inactivated at a low concentration of cysteine (SLUYTERMAN, 1967). However, no inactivation was observed in the lowest concentration studied (3 mM). Burnett et al. (2013) reviewed possible dermal irritation effects caused by α-amino acids. The authors cited that cysteine (and cysteine hydrochloride) can be considered safe in the use of cosmetic products with concentrations up to 5%. Since the 3 mM concentration presented a similar result to the other ones, this concentration was evaluated in the other experiments.

3.3.2 Influence of papain concentration on caseinolytic activity and degree of hydrolysis of casein

Casein is not a protein of the skin. However, this substrate is commonly used to determine the non-specific proteolytic activity of enzymes. Furthermore, the determination of caseinolytic activity was performed to evaluate the activity of papain in a soluble substrate (both collagen and keratin hydrolysis were performed with both substrates as suspensions). It is also important to highlight that the enzymes can present different reactional aspects depending on the substrate. Casein hydrolysis (Figure 3.1a) is obtained by reacting the peptide chains of casein (represented as micelles) with the release of peptides and amino acids.

Table 3.2 shows the effects of different enzyme concentrations on the caseinolytic activity. In general, there was an improvement in caseinolytic activity with the increase of papain. However, no significant difference ($p < 0.05$) was observed when comparing the assays with 0.5 and 1.0 mg mL^{-1} of the catalyst. Moreover, the increase in the caseinolytic activity was not proportional to the increase in papain concentration. The present results were similar to other studies, such as Ji et al. (2011) and Foda et al. (2016). In this last study, for example, the authors demonstrated that the increase of papain concentration improved their results of caseinolytic activity until 0.6 % (v/v) of the enzyme was used.

Figure 3.1: Possible mechanism of hydrolysis of casein (a), collagen (b), and keratin (c) by papain action in aqueous medium.



Source: Trevisol et al. (2022).

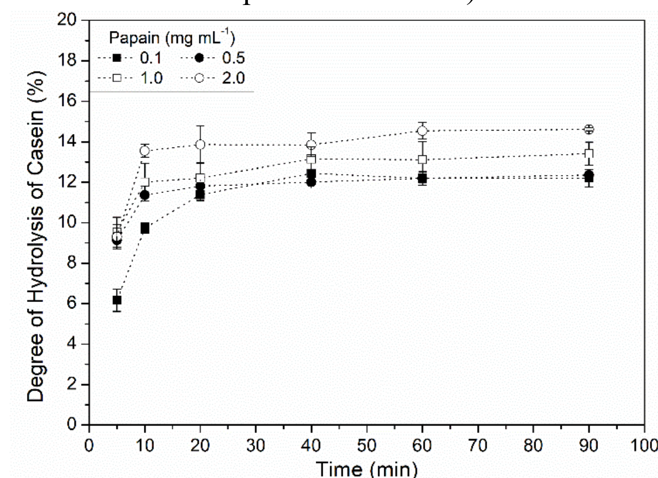
Table 3.2: Effect of papain concentration on the proteolytic activity of casein, collagen, and keratin as substrates.

Papain (mg mL^{-1})	Caseinolytic Activity (PU mL^{-1})	Collagenolytic Activity (CU mL^{-1})	Keratinolytic Activity (KU mL^{-1})
0.1	1.19 ± 0.08 [a]	2.77 ± 0.10 [a]	1.08 ± 0.26 [a]
0.5	1.69 ± 0.06 [b]	4.42 ± 0.82 [b]	1.06 ± 0.19 [a]
1.0	1.98 ± 0.01 [b]	4.34 ± 0.03 [b]	2.42 ± 0.24 [b]
2.0	2.33 ± 0.01 [c]	6.37 ± 0.85 [b]	2.57 ± 0.41 [b]

Assay's condition: 37 °C and pH 7.5. Average ± standard deviation of experimental determinations. Averages with the same letter, in the same column, indicate no significant differences ($p < 0.05$) by the Tukey test.

The effect of time and enzyme concentration was evaluated, and the results of DH of casein are shown in Figure 3.2. The first 10 min of reaction presented a higher DH rate for all concentrations. Within 40 min the reaction appears to have stabilized, reaching 11 – 14% of DH. Similar results of hydrolysis of casein were observed in other studies (KUMAR et al., 2016; LIU; LUO; LI, 2012; ZHAO; WU; LI, 2010)The increase in enzyme concentration tended to improve the DH of casein. Other studies reached DH values close to 20%, but only after 5 h of reaction (KUMAR et al., 2016; LIU; LUO; LI, 2012; ZHAO; WU; LI, 2010). Our results showed that the use of 1.0 and 2.0 mg mL^{-1} of papain give similar casein hydrolysis and may assist the study of the hydrolysis of other soluble proteins.

Figure 3.2: Effect of papain concentration on degree of hydrolysis (DH) of casein (assay at pH 7.5 and 37 °C).



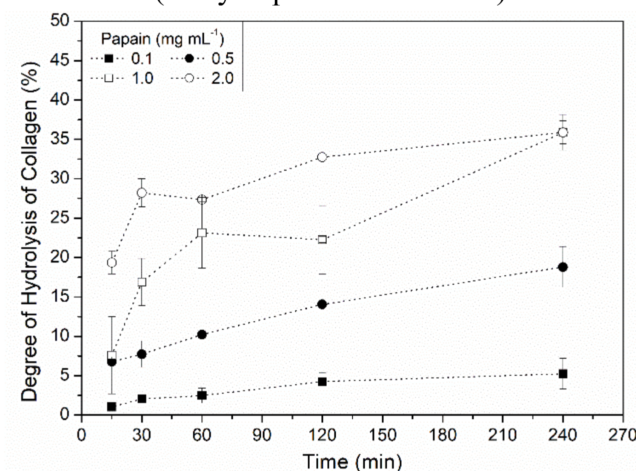
Source: Trevisol et al. (2022).

3.3.3 Influence of papain concentration on collagenolytic activity and degree of hydrolysis of collagen

The collagenolytic activity was also performed in the presence of 3 mM of cysteine (low values were obtained when the amino acid was not added, data not shown). The results of the collagenolytic activity of papain are shown in Table 3.2. As can be observed, the increase of papain concentration did not significantly ($p < 0.05$) affect the collagenolytic activity of papain. The only assay that results in lower ($p > 0.05$) activity was obtained when 0.1 mg mL^{-1} of papain was used. This result probably occurred because, at low papain concentration, lower enzyme-substrate contact (mass transfer effect) occurred or due to product inhibition. After the assays, collagen powder could be observed only when 0.1 mg mL^{-1} of papain was used (data not shown). Different from the results of the present work, Tan, Chang, and Meng (2019)and Shi et al. (2009) observed an increase in the initial rate of hydrolysis of collagen substrates when the authors augment the papain load. The discrepancy may be a result of the cysteine activation, which improved the reaction rate and, consequently, the collagenolytic activity.

The influence of time and papain concentration on the DH of collagen is demonstrated in Figure 3.3. For the 2.0 mg mL^{-1} of papain assay, the DH increased principally until 30 min of reaction, stabilizing it after. The assays using 0.1 and 0.5 mg mL^{-1} of papain reached 5 and 18% of DH at 240 min, respectively After 240 min, 35% of DH was obtained using both 1.0 and 2.0 mg mL^{-1} of papain.

Figure 3.3: Effect of papain concentration on degree of hydrolysis (DH) of collagen (assay at pH 7.5 and 37 °C).



Source: Trevisol et al. (2022).

The collagen hydrolysis by papain (and some other proteases) can be divided into two stages: a faster, when the enzyme acts in the most available cutting sites (probably the non-crosslinked residues) of the collagen; and a slower, reaching steady phase, when the enzyme attacks other protein chains. Figure 3.1b illustrates collagen hydrolysis. The slower activity probably occurs due to the decreased protein chains of collagen that papain can act, product inhibition, or enzyme denaturation (FORGHANI et al., 2012). The two-stage pattern was only observed in the 2.0 mg mL^{-1} assay. Similar DH values and profiles of papain action were observed using different collagen-based substrates (FORGHANI et al., 2012; HA et al., 2012; ILTCHENCO; KEMPKA; PRESTES, 2017; LIN et al., 2017).

The results of collagen hydrolysis are different from those observed when casein was used as substrate. Different from casein, collagen was not solubilized in the reaction medium, which affects the mass transfer and, consequently, the enzyme-substrate contact. Collagen structure itself presents conformational challenges for the enzyme action due to its crosslinked structure. For dermatological use, the 0.5-2.0 mg mL^{-1} concentration seems to provide better results, principally using 2.0 mg mL^{-1} of papain since low contact time is needed.

3.3.4 Influence of papain concentration on keratinolytic activity and degree of hydrolysis of keratin

In general, papain and other proteases present problems in promoting the hydrolysis of keratin. The enzymes preferably act in the extremities or the lateral amino acid residues of the protein chains (LIN et al., 1992). However, the enzymes do not act in disulfide bonding (sulphitolysis) of keratin. Disulfide bond reducing agents can be used to improve the hydrolysis of keratin (NAVONE; SPEIGHT, 2018; RAMNANI; GUPTA, 2007). Figure 3.1c summarizes the hydrolysis mechanism of keratin using disulfide bond reducing agents and papain.

The effects of the addition of some reducing agents can be observed in Table 3.3. The sodium sulfite and cysteine concentrations evaluated were chosen according to skin-use cosmetic and pharmaceutical formulations (BURNETT et al., 2013). A significant increase ($p > 0.05$) of keratinolytic activity was observed when cysteine was added to the reaction. Similar to what was observed during casein hydrolysis, the variation of cysteine concentration did not change the results. This result demonstrates that cysteine

molecules can act as activators and/or reducing agents for keratin hydrolysis reaction by papain. No significant increase ($p < 0.05$) was obtained when only sodium sulfide was added. Similarly, sodium sulfide addition did not present a significant ($p < 0.05$) effect on the papain activity.

Table 3.3: Effect of the addition of reducing agents in the keratinolytic activity of papain.

Reducing Agent	Keratinolytic Activity (KU mL^{-1})
None	1.09 ± 0.19 [b]
3 mM of cysteine	2.45 ± 0.21 [a]
30 mM of cysteine	2.33 ± 0.15 [a]
0.4 % (w v^{-1}) of Na_2SO_3	1.01 ± 0.09 [b]
3 mM of cysteine + 0.2 % (w v^{-1}) of Na_2SO_3	2.77 ± 0.18 [a]
3 mM of cysteine + 0.4 % (w v^{-1}) of Na_2SO_3	2.76 ± 0.13 [a]

Assay's condition: 1 mg mL^{-1} of papain, 37 °C and pH 7.5.
Average ± standard deviation of experimental determinations. Averages with the same letter, in the same column, indicate no significant differences ($p < 0.05$) by the Tukey test.

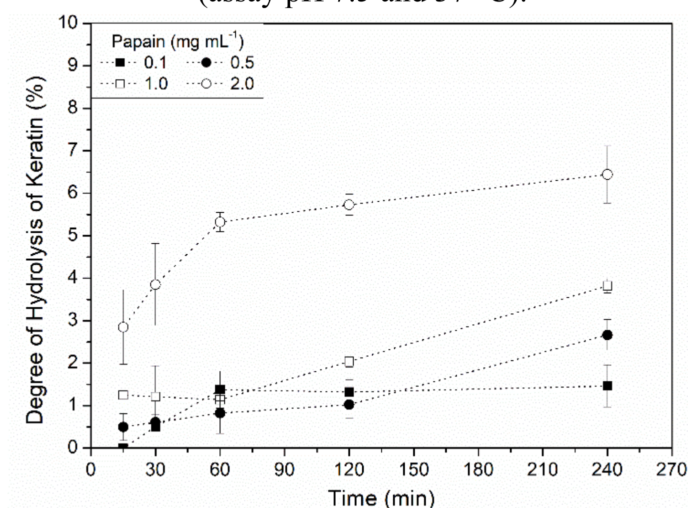
The effects of the reducing agents are dependent on the enzyme, concentration, and reaction time. For example, Ramnani and Gupta (2007) evaluated different reducing agents in the hydrolysis of feather substrate using papain. The authors obtained 60% of hydrolysis when 20 mM of sodium sulfide was used and only 2% of degradation was achieved using cysteine (up to 5 M). However, the reaction time studied was 60 h. Navone and Speight (2018) improved the keratinolytic activity of Ronozyme ProAct by 1.3 and 2.6 times when 1% of sodium sulfide and 2% of thioglycolate were added, respectively. These authors performed the reaction for 1 h. Other concentrations of both reducing agents had negative or no effect. Since no prolonged-time contact is intended to be studied and increase the concentration of the reducing agents, the following experiments were performed in the presence of 3 mM of cysteine.

The keratinolytic activity of papain varying the papain concentration is shown in Table 3.2. The assays with 1.0 and 2.0 mg mL^{-1} did not have a significant difference ($p < 0.05$) among them, but they are superior to the other assays. Only a few studies evaluated the effect of varying the papain concentration on keratin substrates. Jin et al. (2017) demonstrated that the increase of 5 to 20 μg mL^{-1} of papain had improved its keratinolytic activity against feather-substrate by 3 times, approximately. Lennox (1952) evaluated wool hydrolysis by papain, and the author cited that the reduction of enzyme concentration from 2 to 0.2% reduced the digestion of the substrate.

Similar to collagen hydrolysis, the increase of enzyme activity with enzyme concentration (Table 3.2) probably occurred due to the improvement of the contact of papain and the cutting sites of keratin that occurred with more enzymes present in the medium. The no significant difference ($p < 0.05$) results when comparing 1.0 and 2.0 mg mL^{-1} (or 0.1 and 0.5 mg mL^{-1}) can be attributed to reaching all the accessible cutting sites that the enzyme can act in the time or to product inhibition.

Figure 3.4 shows the time and papain concentration on DH of keratin. There was an improvement in DH with time for all assays, except when 0.1 mg mL^{-1} of the enzyme was used. Generally, the incubation time prolongation improves the hydrolysis of keratin substrates by papain, principally when several hours were evaluated (HARA et al., 2014; RAMNANI; GUPTA, 2007). The increase of DH by increasing the enzyme and time can be related to the action of cysteine molecules, which reduces the keratin as the papain acts in the cutting sites until there are no more reducing or cutting sites to both molecules act. The assay using 2.0 mg mL^{-1} of papain resulted in higher DH, reaching 6% in 240 min, whereas less than 5% of DH was reached with lower papain concentration.

Figure 3.4: Effect of papain concentration on degree of hydrolysis (DH) of keratin (assay pH 7.5 and 37 °C).



Source: Trevisol et al. (2022).

3.3.5 Effect of pH on the proteolytic activity of papain with casein, collagen and keratin as substrates

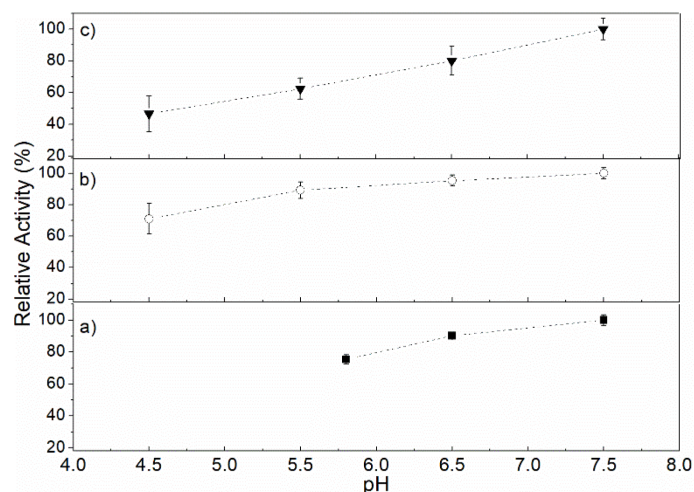
Enzymes are dependent on pH, presenting activity in a specific pH range (BAHRAMI; HOMAEI, 2018). Papain presents pH stability in the range of 5.0 – 7.0,

obviously depending on the substrate and other conditions used (MESHRAM et al., 2019). In the present study, the importance of pH variation can be related to two different aspects. The first is related to the pH of the skin. The pH of the skin varies on various factors, such as age, body location, gender, presence of diseases, etc. The surface of the skin is known to have an acid environment named the “acid mantle.” The pH of the stratum corneum of human skin is below 5.0. The pH increases as the deep of the skin is increased: after 100 µm of the stratum corneum, the pH becomes alkaline and can reach near 8.0 value until 1000 µm (WAGNER et al., 2003). The second important aspect is the pH of the formulation that contains the papain, which can directly affect the enzyme action. In general, cosmetic formulations present pH about 7.0 (BANCHHOR; SARAF, 2008) To reduce the loss of enzyme activity, modification of the enzyme can be performed to minimize this effect in topical formulations, as already demonstrated for papain (PINTO et al., 2011).

Figure 3.5 shows the effect of pH levels of skin on papain activity. The pH decrease tended to reduce the papain activity for all three substrates, principally for keratin. While caseinolytic and collagenolytic activities were maintained at 90%, approximately, the activity of papain on keratin hydrolysis reached 80% at pH 6.5. The highest reduction was observed at pH 4.5, where keratinolytic activity reached 46% against 71% for collagenolytic activity. Similar effects of pH were previously cited for caseinolytic activity (HOMAEI et al., 2010; LI; XING; DING, 2007; SAIKIA et al., 2019). It is important to mention that the solubilization of casein below pH of 6.0 is very difficult. For this reason, in the present work, the pH was adjusted to 5.8 instead of 5.5 as intended. Lennox (1952) described that cysteine-activated papain digested 40% of wool keratin at pH 7.5; when the pH was reduced to 5.5, less than 10% of the substrate was digested. No study evaluating the effect of the collagenolytic activity of papain with pH variation was found.

The pH of the skin can be modified by using water, cosmetics, detergents, and soaps, without damaging the tissue. Then, naturally, the pH value returns to its original value (KORTING; BRAUN-FALCO, 1996). In this sense, the application of a formulation that increases the pH of the skin before the use of papain is recommended to improve the enzyme action. The obtained results demonstrate that, preferably, the formulations containing papain need to present pH from 6.5 – 7.5 during its action in the skin to preserve the proteolytic activity.

Figure 3.5: Effect of pH on proteolytic activity papain on casein (a), collagen (b), and keratin (c) substrate (assays at 37 °C and 1 mg mL^{-1} of papain).



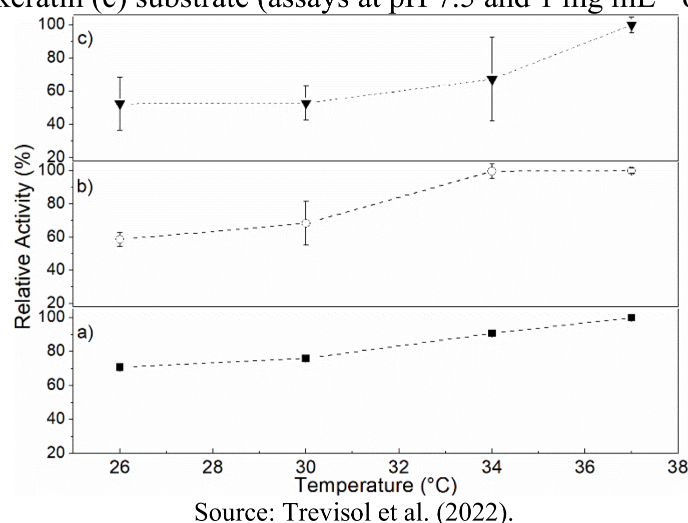
Source: Trevisol et al. (2022).

3.3.6 Effect of temperature on the proteolytic activity of papain with casein, collagen and keratin as substrates

The enzymes present a specific range of temperature to act (BAHRAMI; HOMAEI, 2018). Generally, papain presents a temperature range of 20 – 80 °C, but this is affected by papain type, and substrate used (HOMAEI et al., 2010; SAIKIA et al., 2019). The temperature of the skin varies due to age, body region, presence of curvature regions, the extremities regions of the body, presence of other organs, veins, arteries, fat, muscles, and some diseases (BIERMAN, 1936). Normally, the temperature of the surface of this organ varies from 32 – 35 °C; however, the environment can increase or reduce these values (LAI; ZHOU; CHEN, 2017).

Figure 3.6 shows the influence of temperature on the casein, collagen, and keratinolytic activities. The diminution of temperature media reduced the enzyme action, principally at 30 and 26 °C. Similarly, Errasti et al. (2020) observed that the temperature variation from 35 to 25 °C decreased the collagenolytic and keratinolytic activity of proteolytic preparations obtained from the latex of *C. papaya*. Moreover, the casein hydrolysis by papain tends to be affected by reducing the temperature of the medium (HOMAEI; SAMARI, 2017; SAIKIA et al., 2019). Collagen and keratin presented more proteolytic activity loss among the proteins than casein. It probably occurred because papain presents more specificity or the non-skin substrate than the other two.

Figure 3.6: Effect of temperature on proteolytic activity papain on casein (a), collagen (b), and keratin (c) substrate (assays at pH 7.5 and 1 mg mL^{-1} of papain).



Source: Trevisol et al. (2022).

If possible, it is important to warm the skin region that will future be treated by the enzyme action before its application when using the enzyme for cosmetic or pharmaceutical use. This step can be performed with water, cream, or other solutions applied to the skin. Another important factor is the environment temperature, which must preferably be not too low or high to prevent enzyme activity loss.

3.3.7 Visual aspect of the topical application of papain on skin substrate

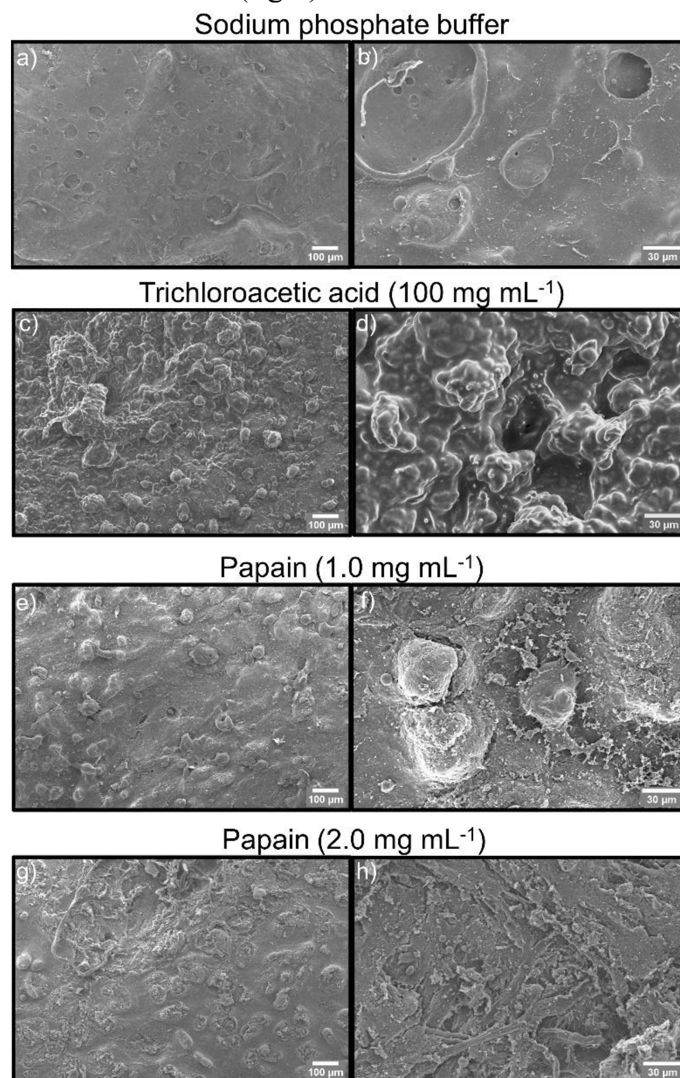
The results of the application of papain (1.0 and 2.0 mg mL^{-1}), TCA (100 mg mL^{-1}), and phosphate buffer solutions (100 mM, pH 7.5) on porcine skin fragments were shown in Figure 3.7. TCA was evaluated as a positive control. This acid is used as a superficial peeling agent when applied at 10 – 25 % (wt.) concentration and the treatment is considered safe and stable (SAFOURY et al., 2009).

It is known that the skin surface is not homogeneous and that the removal technique and storage can influence the appearance of this organ. As a reference, Figure 3.7a shows that the control sample presented a compact, continuous, and low porous structure. At 500x resolution (Figure 3.7b), dethatched fragments can be observed along with the sample, which probably occurred due to the handling or storage.

Differently of the homogenous structure of control, the application of TCA modified the structure (Figure 3.7b and Figure 3.7d). The micrograph showed the appearance of disrupted structures and pores, indicating action on collagen structures. Figure 3.7d shows some disruptions on the skin, possibly indicating that TCA hydrolyzed

some layers of the skin and left collagen exposed. When Sifaki et al. (2019) compared the micrographs of skin specimens before and after needle shaping treatment, the authors obtained similar structures to the present work.

Figure 3.7: Scanning electron micrographs of the surface of porcine skin fragments after the application of sodium phosphate buffer (a, b), trichloroacetic acid (TCA, c, d), 1.0 (e, f), and 2.0 mg mL^{-1} (g, h) papain solutions. Magnificence of 100x (left) and 500x (right) are shown.



Source: Trevisol et al. (2022).

The topical application of papain modified the surface of the skin. When 1.0 mg mL^{-1} of protein solution was used (Figure 3.7e), there was an appearance of irregular and rougher structures, indicating the hydrolysis of proteins. At 500x resolution (Figure 3.7f), it was clear that the enzyme exfoliates the skin by observing the removal of the more compact structure and appearance of detached and fibrils proteins. The effect of

the removal of proteins was intensified when 2.0 mg mL^{-1} of papain was used (Figure 3.7g and 3.7h) and corroborates with the results of keratin hydrolysis. It does not seem that papain action promoted the hydrolysis of collagen fibers, acting only in the epidermis. The obtained results are similar to those observed by the SEM micrographs of Errasti et al. (2020) when the authors applied proteases from the latex of *C. papaya* on cowhide samples.

3.4 CONCLUSION

Papain is a well-studied and used plant protease that has numerous applications. Nevertheless, there are still lacunes regarding the use of this enzyme to hydrolyze proteins of the skin and the effect of varying its final concentration and use of activators in this type of application.

The present study reports the importance of evaluating papain final concentration and the addition of cysteine as an activator when formulating pharmaceutical and/or cosmetic products to act in the human skin. Based on the results of this work, the addition of 3 mM of cysteine improved the proteolytic activity of papain with casein, collagen, and keratin being used as substrates. In addition, the use of 2.0 mg mL^{-1} of papain achieved the highest values of DH at low contact times, results that are interesting for pharmaceutical and cosmetic applications. The enzyme also was able to maintain its activity at conditions on the surface of human skin (34 °C and pH of 5.0). The SEM micrographs indicate that papain can exfoliate the external layers of the skin and can be used as a transdermal drug permeation enhancer or skincare exfoliant. The data of this study contribute to the better knowledge of the papain use on the condition of human skin to cosmetic and pharmaceutical use in topical and transdermal formulations.

CHAPTER 4: STARCH- AND CARBOXYMETHYL CELLULOSE-BASED FILMS AS ACTIVE BEAUTY MASKS WITH PAPAIN INCORPORATION

This section is based in related to the second experimental article. This article was published in International Journal of Biological Macromolecules as “*Starch- and carboxymethyl cellulose-based films as active beauty masks with papain incorporation*”. This article was accepted in 2023 and indexed (https://doi.org/10.1016/j.ijbiomac.2023.123258). According to Elsevier subscription rules, the authors retain the right to include the article in a thesis, provided it is not published commercially.

4.1 INTRODUCTION

The cosmetic sector is a growing market with a wide variety of products (PACHECO et al., 2018). From a historic perspective, face masks are one of the oldest cosmetic products still in use nowadays (PAN-ON et al., 2018). This type of cosmetic is traditionally known to moisturize, white, bright, and clean the skin's pores. With the advancement of technology and the discovery of new compounds, the face mask changed to new aspects and ingredients, increasing their benefits and use (NILFOROUSHZADEH et al., 2018). The use of face masks in the skincare routine is considered excellent, effective, and easy to be performed at home (AFONSO et al., 2019).

Beauty face masks can be divided into creams, clays, and sheets (ZHAO et al., 2019). Sheet masks are thin face-shaped materials applied alone or combined with other cosmetics to cover the face region (PERUGINI et al., 2019). The growth of this type of mask influenced the increase in the sales of personal care products. Different polymers can be used to produce sheet masks. Ecofriendly and bio-derived materials are preferred by customers (MORGANTI et al., 2020). Among the biopolymers, chitosan, cellulose, alginate, and starch are already studied to produce face masks (AFONSO et al., 2019; HE et al., 2018; PACHECO et al., 2018; SURINI; AULIYYA, 2017).

Starch is a natural polymer obtained from diverse food stocks in nature. Generally, the gelatinization process reduces the semi-crystalline structure of starch, which improves the properties and processability of the polymer (COLTELLI et al., 2018; RATNAYAKE; JACKSON, 2008). Starch films generally have good film-forming and

barrier properties but lack mechanical properties and water resistance (TAVARES et al., 2019). Blending starch with other compatible polymers may overcome the undesired characteristics of the material. Carboxymethyl cellulose (CMC) is an anionic polysaccharide derived from cellulose that is known to form highly flexible and water-soluble films (TREVISOL et al., 2019). Films of the blend of starch and CMC seem to have high hydrophilicity, water-holding capacity, strength and flexibility to mold the skin, low toxicity, and biodegradability (GHANBARZADEH; ALMASI; ENTEZAMI, 2010; GHANBARZADEH; ALMASI; OLEYAEI, 2013; PUTRI; SETIAWAN; ANGGRAINI, 2017; TAVARES et al., 2019; VERONESE et al., 2018). Moreover, the blend of both polymers can be cross-linked by citric acid to reduce the possible excessive water solubility of the material (GHANBARZADEH; ALMASI; ENTEZAMI, 2010).

Different active ingredients can be incorporated into face masks to bring their properties to the cosmetics, such as exfoliants (NILFOROUSHZADEH et al., 2018). Exfoliating agents can promote and accelerate the desquamation process of the skin by removing the outmost layers of the skin. Among the exfoliating agents, enzymatic exfoliation is based on applying proteolytic enzymes that hydrolyze the skin's proteins. Consumers have been looking for enzymatic exfoliation as it is a more sustainable option with a more specific and less aggressive action than other types of exfoliants. Papain is the most studied enzyme to act as an exfoliant (TREVISOL et al., 2021). This enzyme is extracted from papaya (*Carica papaya*) and has consolidated industrial use in different sectors, including the cosmetic industry (AMRI; MAMBOYA, 2012).

Although there are already studies that evaluated the immobilization of papain in polymeric films/membranes (ASANARONG et al., 2021; DUTRA et al., 2017; MOREIRA FILHO et al., 2020; RAVINDRA; SARASWATI; BHAGYAVANA, 2013; SHOBA et al., 2014; SILVA et al., 2016; VASCONCELOS et al., 2020; WONGPHAN et al., 2022), no research, to our knowledge, investigated the immobilization of this enzyme in films of the blend of starch and CMC or related to the production of active beauty face masks. In this sense, this work aims to evaluate the influence of the incorporation of papain in the structure and physico-chemical properties of films of starch and CMC to act as beauty face masks. Moreover, the proteolytic activity and stability of the films were also investigated to demonstrate if the materials could preserve the enzyme activity after the immobilization and are stable during storage.

4.2 MATERIAL AND METHODS

4.2.1 Material

The polymers potato starch (Shambala Naturais, Brazil) and medium-viscosity sodium carboxymethyl cellulose (degree of substitution 0.6, Neon, Brazil) were used to produce the films. The citric acid (Neon, Brazil) was used as a cross-linking agent and glycerol (Sigma-Aldrich Chemical, United States) as a plasticizer. Papain from *Carica papaya* (activity ≥ 3.6 units mg^{-1} protein) was purchased from Sigma-Aldrich Chemical Co. (USA). Sodium casein (Dinâmica Química, Brazil), skin sections of freshly slaughtered pigs (obtained from a local slaughterhouse), sulfanilic acid, trichloroacetic acid (Neon, Brazil), and tyrosine (Synth, Brazil) were used to determine the proteolytic activities. SYPRO™ Ruby Protein Gel Stain (Invitrogen, USA) was used as a dye agent in confocal microscopy. All the other reagents were of analytical-grade quality.

4.2.2 Film preparation

The films were produced by the casting method (DUTRA et al., 2017; GHANBARZADEH; ALMASI; OLEYAEI, 2013). Starch (5% (w/v)) was dissolved at room temperature under mechanical stirring at 900 RPM (RW 20, Ika, GE) in distilled water. Then, 0.5% (w/v) of citric acid was dissolved into a starch filmogenic solution. The mixture was heated to 80 °C, maintaining the mechanical stirring for 30 min for the gelatinization and cross-linking processes to occur. Then, the starch filmogenic solution was cooled at room temperature. Separately, different amounts of papain were dissolved in distilled water at room temperature at 400 RPM. Then, 1% (w/v) of CMC was dissolved in each papain solution under mechanical stirring. The final enzyme concentrations were 0, 1, 2, 5, 10, and 20% (w/w, g of papain per g of total polymer in the films after the mixture); the films were named, respectively, P0, P1, P2, P5, P10, and P20.

Starch- and CMC- and papain-filmogenic solutions were mixed at room temperature under mechanical stirring (600 RPM) at a 1:1 (w:w) polymeric ratio. Glycerol (1.15% w/v) was added to the mixtures, and the mixtures were maintained at room temperature overnight. Then, 225 g of the solution was poured into acrylic plates (25 cm^2) and dried in a fume hood for 36 h at room temperature. The films were

dethatched from their supports and conditioned at 58 ± 2% relative humidity for at least 48 h before any characterization or use.

4.2.3 Physic-chemical characterization of the films

The visual macroscopic aspect of the films was determined with a photographic camera (model DSLR D5500, Nikon, Japan), considering homogeneity, handling, and presence of any visual grumes. The microscopic aspect of the films was evaluated with a scanning electron microscope (model JSM-6390LV, JEOL, Japan) with an accelerating voltage of 10 kV. The samples were placed on stubs and coated with an ultrathin gold layer (model SCD500, Leica, Germany).

The average thickness of the films at 10 random positions was used as the mean thickness of each film. A digital micrometer (MDC-25P, Mitutoyo, Japan) was used to measure the thickness of the films (n = 8). Specimens with any visible defects were discarded.

The films' moisture content (MC) was determined gravimetrically by air drying the samples at 105 °C for 24 h. MC values were expressed as g of water per 100 g wet material.

The sessile drop method measured the water contact angle (WCA). The films (1 cm × 2.5 cm), previously fixed on glass slides to provide better stretch, were attached to the goniometer (Ramé-Hartz 250, Ramé-Hartz Instrument Co., Germany). Then, one drop (40 µL) of distilled water was released over the surface of each film using an automatic precision syringe. The DROPimage Advanced software was used to calculate the contact angle between the film and the drop. Five measurements for each film (n = 4) were taken.

The solubility matter (SM) of the films was also determined gravimetrically. Four samples (5 cm^2) were weighed (W_i) and immersed in 40 mL of deionized water for 24 h at 37 ± 3 °C. Then, the films were dried at 105 °C for 24 h and weighed again (W). SM was calculated by Equation (4.1).

$$\text{Solubility Matter (SM)} = \frac{W_i - W}{W_i} \times 100 \quad (4.1)$$

The tensile strength and elongation at break were determined at room temperature according to ASTM standard method D88-02 (ASTM D88-02, 2002). The

tests were performed using the crosshead speed of 50 mm/s, initial grip spacing of 60 mm, and load cell capacity of 50 N (TA.HD.plus Texture Analyzer, Stable Micro Systems UK). Eight rectangular samples of each film measuring 90 mm x 25 mm were used.

The attenuated total reflectance Fourier Transform Infrared (ATR-FTIR) spectra of the films was recorded to investigate the chemical composition and possible interactions between the components of the films. FTIR spectrometer (Cary 660, Agilent Technologies, USA) was used in the measures with a wavenumber range of 4000–700 cm^{-1} and resolution of 4 cm^{-1}.

4.2.4 Evaluation of protein distribution along with the films

The papain release of the films was measured to determine how the enzyme was distributed along with the films. Films were cut into pieces of approximately 2.8 cm^2. Then, the samples (n = 3) were immersed in 50 mL of distilled water and incubated in an orbital shaker (TE 424, Tecnal, Brazil) for 24 h at 30 ± 3 °C and 120 RPM to facilitate the release of the enzyme. The Lowry method determined the protein content of each sample (LOWRY et al., 1951).

The protein distribution was observed by confocal microscopy. The samples were immersed in the solution of SYPRO™ Ruby Protein Gel Stain for 20 min and washed with phosphate sodium buffer (pH 7.4. 50 mM) to remove the dye excess. The images were obtained with a confocal laser scanning microscope (CLSM, model DMI6000 B, Leica) with Excitation/Emission of 560/590. The obtained images were treated in the ImageJ software.

4.2.5 Enzymatic activity of the films using casein as substrate

The non-specific proteolytic activity of the films was determined according to Homaei et al. (2010) using casein as substrate, with some modifications. Briefly, 0.5 mL of casein solution (0.65% w/v, pH 7.5) was mixed with 0.5 mL of 100 mM phosphate buffer (pH 7.5) in 2 mL centrifuge microtubes. Then, 0.25 g of the films were put into the microtubes and conditioned at 34 °C for 20 min. The reaction was stopped by adding 0.5 mL of 10% (w/v) of TCA solution and removing the films. The blanks were performed similarly, but the TCA solution was added prior to the films. The microtubes were centrifuged for 5 min at 8,170 xg, and the absorbance of the supernatant was observed

spectrophotometrically (U-2900, Hitachi, Japan) at 280 nm. The results were correlated to a tyrosine standard curve. One unit of caseinolytic activity (U_{casein}) was defined as the amount of enzyme required to liberate 1 μmol of tyrosine per minute under the defined assay conditions.

4.2.6 Enzymatic activity using skin as substrate

The proteolytic activity of the films was also determined using skin as a substrate. Firstly, porcine skin was cut into fragments of approximately 30 $cm^{2,}$ and the excess fat was manually removed with the help of scissors. The obtained fragments were washed with deionized water and dried for 24 h at 40 °C. Then, the skin was milled with a food processor, resulting in approximately 0.1 cm2 fragments. The lipids of the skin were extracted according to Cequier-Sánchez et al. (2008), with modifications. The milled skin was immersed in a solution of dichloromethane and methanol (2:1 volumetric ratio). At room temperature, the suspension was maintained at 100 RPM agitation for 2 h. Then, the mixture was filtered, and the skin was washed with deionized water five times to remove the excess solvent. The obtained skin fragments were freeze-dried.

The azo skin substrate was produced by dying the freeze-dried skin fragments with sulfanilic acid, adapted from Gonzalo et al. (2020). The obtained substrate was named azo porcine skin substrate (APSS) and used to determine the enzymatic activity. Briefly, 0.2 g of APSS was added to Erlenmeyer flasks of 50 mL. Then, 30 mL of sodium phosphate buffer solution (100 mM, pH 7.5) was added, followed by adding 1.25 g of the films. The reaction was maintained at 125 RPM of agitation at 34 °C for 1 h in an orbital incubator (TE-424, Tecnal, Brazil). The reaction was stopped by removing the films and placing the flasks in a cold-water bath for 10 min. Finally, the samples were centrifuged (10 min at 8,160 xg). The control samples were performed similarly but without adding the films. The absorbance of the supernatant was analyzed at 415 nm with a spectrophotometer (Spectro S-2000, BEL, Italy). One unit of enzymatic activity (U_{APSS}) was defined as the amount of papain yielding 0.01 absorbance units/h.

4.2.7 *In vitro* evaluation of the exfoliation activity of the films

The evaluation of exfoliation activity of the films was performed similarly to our previous work (TREVISOL et al., 2022), with some modifications. Briefly, porcine skin

fragments were obtained by manually removing the excess fat with tweezers and scissors and washing the samples with distilled water. The skin was cut into pieces of 2 cm^2 (approximately) and dried at room temperature for 24 h. Then, 75 µL of sodium phosphate buffer (100 mM, pH 7.5) was added over the skin to hydrate and maintain the pH of the region to the desired one. The films were put over the skin to cover the fragments and maintained at 37 ± 3 °C for 30 min. After the incubation, the films were removed and the skin pieces were washed by immersing them in a phosphate buffer solution.

The skin fragments were immersed in a 2.5 % (w v^{-1}) glutaraldehyde-phosphate buffer solution (pH 7.4) for 2 h. After the fixation, the skin fragments were washed in phosphate buffer to remove the excess glutaraldehyde. The samples were pos-fixated, immersing them in a 1.0 % (w v^{-1}) osmium tetroxide-phosphate buffer solution (pH 7.4) for 2 h. The samples were washed with distilled water to ensure the removal of all unbound osmium tetroxide molecules. Then, the samples were dehydrated by immersing them in increasing ethanol concentrations and dried at the critical point (EM CPD 030, Leica, Germany). The dried fragments were placed on stubs, coated with an ultrathin gold layer, and analyzed with scanning electron microscopy.

4.2.8 Stability test

An accelerated stability test was performed for the films P0, P1, P2, and P5 in accordance to the standards of the Brazilian Health Surveillance Agency (ANVISA (AGÊNCIA NACIONAL DE VIGILÂNCIA SANITÁRIA), 2004). The films were stored in transparent glass containers at three different temperatures: 6 ± 2 (refrigerator), 22 ± 5 (room temperature), and 37 ± 3 °C (oven). The tests were conducted at 0, 1, 7, 15, 30, 60, and 90 days. The relative humidity of the storage conditions was maintained at 58 ± 2% for room and oven temperatures. The visual aspect, moisture content, and enzymatic activity (casein as substrate) were evaluated (n = 4).

The results of the remaining caseinolytic activity in the films during the stability test were demonstrated as Relative Activity. This parameter was calculated according to Equation (2), where Activity is the activity determined at a certain time storage condition, and Initial Activity was the activity measured before the first day of storage.

$$Relative\ Activity\ (\%) = \frac{Activity}{Initial\ Activity}\ x\ 100 \quad (4.2)$$

4.2.9 Statistical analysis

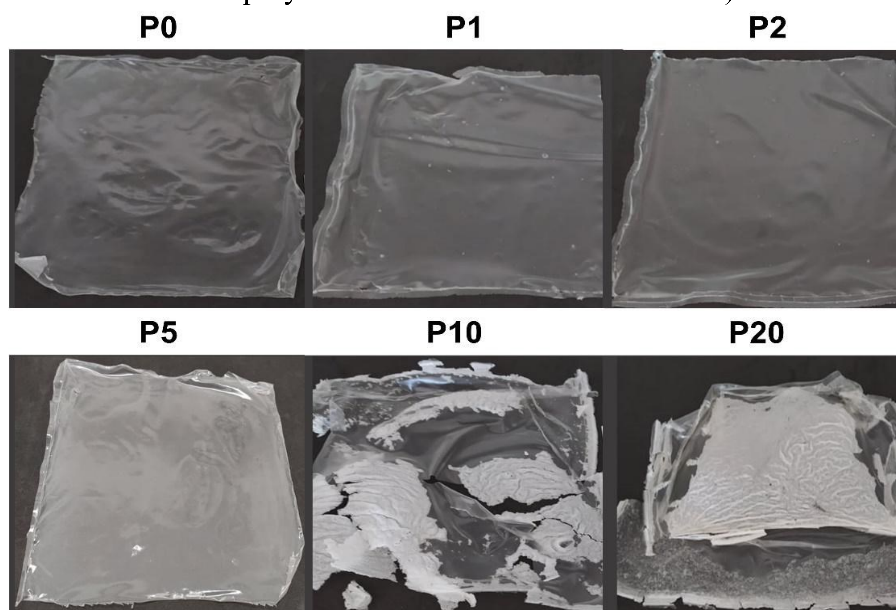
Analysis of variance (ANOVA) and Tukey test was used to statistically determine the significant differences ($p < 0.05$) among averages using the software STATISTICA (version 7.0). According to the Tukey test, averages with the same subscript letter in the same column had no significant differences ($p < 0.05$).

4.3 RESULTS AND DISCUSSION

4.3.1 Morphology and protein distribution

Figure 4.1 shows the macroscopic visual of the films. Before drying, all filmogenic solutions did not present any undissolved particles (data not shown). However, the increase in papain concentration affected the appearance of the films. P10 and P20 films were heterogeneous and brittle and difficult to detach from the molds. Whitish fibrous structures were observed in these films and can probably be correlated to papain's supersaturation during drying. For this reason, both formulations were discarded for further characterization.

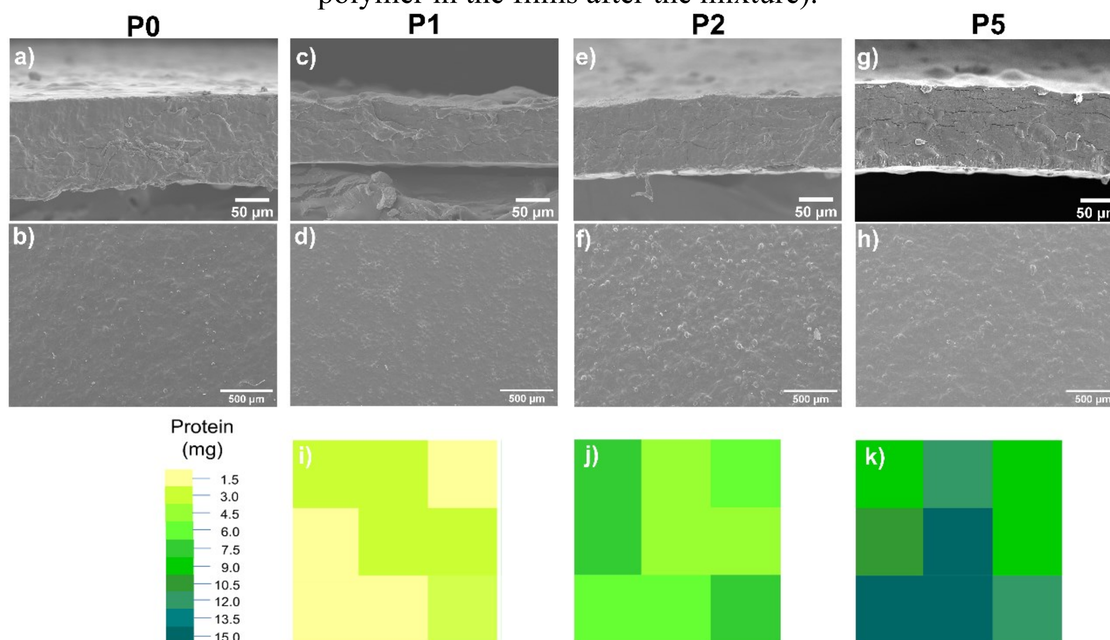
Figure 4.1: The visual aspect of the films containing the final papain concentrations P0 (0%) , P1 (1%), P2 (2%), P5 (5%), P10 (10%) and P20 (20%) (w/w g of papain per g of total polymer in the films after the mixture).



The visual aspect of the P1 and P2 films was similar to the control film (P0). On the other hand, the P5 film presented a whitish aspect but was considered homogenous. This whitish aspect was also observed in the study of Dutra et al. (2017) when the authors incorporated 2% (w/v) of papain in their alginate- and polyvinyl alcohol-based films. In general, beauty face masks were traded in different textures and colors. However, a new tendency of invisible and thin masks was observed in recent years (ZHAO et al., 2019), indicating that the studied masks are interesting for the cosmetic market.

The scanning electron micrographs and protein distribution of the films are shown in Figure 4.2. In general, all films presented similar microstructure regarding their cross-sections (Fig. 4.2a, 4.2c, 4.2e, and 4.2g), with no appearance of agglomerates or particles. On the other hand, the incorporation of papain changed the surface of film P0 (with the emergence of some protuberances, principally for P2 and P5 films (Fig. 4.2f and 4.2h, respectively) when compared to control (Fig. 4.2b). In general, no cracks were observed on the surface of the films. The addition of the enzymes did not tend to cause any signal of degradation in the matrix.

Figure 4.2: Scanning electron micrographs of the cross-section (a, c, e and g), surface (b, d, f and h) and papain distribution (I, j and k) of the films containing the final papain concentrations P0 (0%), P1 (1%), P2 (2%), and P5 (5%) (w/w g of papain per g of total polymer in the films after the mixture).

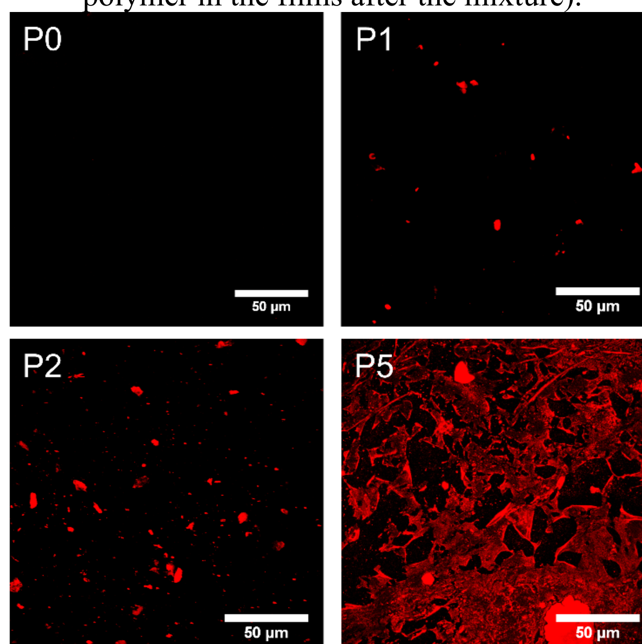


Figures 4.2i, 4.2j, and 4.2k show the papain distribution obtained by the protein release in liquid media for P1, P2, and P5 films, respectively. The amount of papain

released depended on the initial papain addition: 34, 40, and 37% of the total protein initially added was released from films P1, P2, and P5, respectively. This result indicates that part of the papain that was incorporated had a structure function and was entrapped into the polymeric matrix, providing a physical barrier to mass transfer. This result shows how the papain was distributed along all films. Each film was divided into nine quadrants, and each quadrant had the presence of the enzyme. These results indicate that when using the films as beauty face masks, all regions of the face could present the desired enzymatic activity, which is interesting for this type of application. It is interesting to mention that the temperature variation could lead to different release patterns, but the temperature was chosen accordingly to the beauty face mask use.

The macrographs of confocal microscopy are shown in Figure 4.3. As expected, the increase in initial papain amount for each film resulted in observing a higher protein on the films. P5 film seems to be covered with papain, while in P1 film, only some parts had the enzyme. Moreover, in the P5 film, some protein agglomeration can be observed in the micrographs, a result that is not observed in P2 and P1. As a control, the P0 film did not show any protein signal.

Figure 4.3: Confocal micrographs of the surface of the films containing the final papain concentrations P0 (0%), P1 (1%), P2 (2%), and P5 (5%) (w/w g of papain per g of total polymer in the films after the mixture).



4.3.2 Thickness, moisture content, water contact angle, and solubility matter of films

There is no significant difference ($p < 0.05$) between the thickness of the films (Table 4.1). These results can be related to the low amount of papain added in the formulation compared to the amount of polymer, which was probably insufficient to affect this parameter. The values of thickness observed in Table 4.1 agree with the results observed by micrograph images of the cross-section of the films (Figures 4.2a, 4.2c, 4.2e, and 4.2g).

Table 4.1: Thickness, moisture content (MC), water contact angle (WCA), and solubility matter (SM) of films containing the final papain concentrations P0 (0%), P1 (1%), P2 (2%), and P5 (5%) (w/w g of papain per g of total polymer in the films after the mixture).

Film	**Thickness (µm)**	**MC (%)**	**WCA (°)**	**SM (%)**	**Film**	**SM (%) ***
P0	167.9 ± 25.7 ^{a}	14.0 ± 0.9 ^{a}	41.3 ± 3.5 ^{b}	20.8 ± 1.8 ^{b}	P0$^{\#}$	29.78 ± 7.6 ^{a}
P1	149.3 ± 19.9 ^{a}	12.2 ± 1.1 ^{a}	62.4 ± 9.1 ^{a}	12.4 ± 1.0 ^{a}	P0*	17.3 ± 2.1 ^{b}
P2	155.2 ± 7.6 ^{a}	13.3 ± 0.3 ^{a}	67.0 ± 3.7 ^{a}	12.0 ± 3.4 ^{a}	P1*	13.4 ± 3.3 ^{b}
P5	159.8 ± 30.1 ^{a}	12.0 ± 0.5 ^{a}	64.1 ± 2.8 ^{a}	16.9 ± 4.4 ^{ab}	P2*	11.4 ± 0.6 ^{b}

* Effect of the absence of citric acid and/or glycerol on the SM of the films. $^{\#}$: indicate the composition equal to the film but without citric acid. Means in the same column followed by different subscript letters are significantly different ($p < 0.05$).

The MC of all films varied between 12 and 14% (Table 4.1) without significant difference ($p < 0.05$). This result suggests that papain has a similar hygroscopic characteristic compared with starch and CMC or that the enzyme concentration was insufficient to affect this characteristic.

From Table 4.1, all films presented a partially wettable surface (30° < WCA < 90°), which is desired for skin applications (DUTRA et al., 2017), such as beauty masks.

The incorporation of papain increased ($p > 0.05$) the WCA values of the films. The results can be explained by the fact that papain presents hydrophobic amino acid residues (AMRI; MAMBOYA, 2012), which probably appeared along with the films. However, no difference was observed with the enzyme increase in the films. For example, our results differ from Dutra et al. (2017). The authors observed a reduction of WCA when papain was added to their alginate PVA- and alginate-based films. The authors explained the results by roughness surface that occurred with papain addition, which probably is not so considerable in the current study.

The evaluation of the solubility of the beauty masks is useful to demonstrate if the materials maintain their integrity when in contact with liquid solutions that can be applied simultaneously to the skin (AFONSO et al., 2019). In general, all films presented low SM values (Table 4.1), demonstrating that these materials are interesting candidates for applying as beauty masks. Although both CMC and starch-based films present high solubility in water, blending both polymers tend to produce films with low SM values (GHANBARZADEH; ALMASI; ENTEZAMI, 2010; PUTRI; SETIAWAN; ANGGRAINI, 2017; TONGDEESOONTORN et al., 2011).

The addition of papain affected SM values of the films (Table 4.1). P1 and P2 films presented lower SM ($p > 0.05$) than the P0 film, demonstrating that the low amount of papain produced a more compact and concise matrix, reducing the possible hydrolytic degradation of the polymeric matrix. On the other hand, no significant difference ($p < 0.05$) was observed between the P0 and P5 films for SM. This result can indicate that the excess of papain present in P5 film led to a not so concise matrix and can be related to the supersaturation spaces that appeared in the visual aspect (Figure 4.1), which could facilitate the film solubilization. The results of SM demonstrated that all formulations could be used as beauty masks without any significant loss when applying other components with the films or when hydrating the mask or the skin area before using the product.

Another interesting aspect to be correlated with SM is the barrier property. In general, it seems that the incorporation of papain did not affect the moisture migration property of polymeric films, even if there is an alteration of SM with enzyme addition. For example, Wongphan et al. (2022) observed that the papain addition of up to 15% provided an insignificant effect on the water vapor permeability of their starch-based films. Similarly, the results of the work of Santos et al. (2021) indicated that the incorporation of up to 10% of papain did not affect the water vapor permeability of their

chitosan-based films. The results indicate that the major effect in moisture migration property is related to the polymeric matrix itself and not to amino acid residues which papain contains.

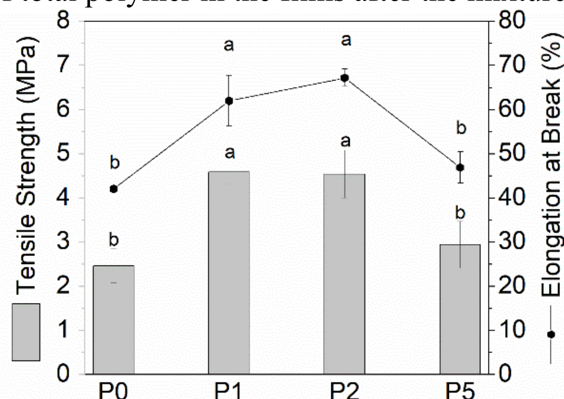
Films without citric acid and/or glycerol were also prepared to investigate if papain had a cross-linking property or if these film components had a synergistic effect in this parameter. The obtained results (Table 4.1) demonstrated that citric acid addition reduced solubility matter, proving the cross-linking effect of this agent ($P0^{\#}$ film). Moreover, papain and critic acid both influenced the solubility of the films, which did not occur when only papain was added (comparing $P0^{*}$ and $P1^{*}$/$P2^{*}$ films). The results indicate that citric acid cross-linked the polymeric matrix of the films and that papain also presented this characteristic.

4.3.3 Mechanical properties and chemical structure

The results of the mechanical properties of the films are shown in Figure 4.4. The incorporation of papain improved ($p > 0.05$) both tensile strength and elongation at the break of P1 and P2 films compared to the control assay. Similarly, the increase of elongation at break (DUTRA et al., 2017) and tensile strength and elongation at break (RAVINDRA; SARASWATI; BHAGYAVANA, 2013) were observed from other studies that evaluated the addition of papain in films of alginate and PVA and PVA and chitosan, respectively. The obtained results are not all well elucidated but indicate that papain provided plasticizing and reinforcing effects on the polymeric matrix in films P1 and P2.

The mechanical properties corroborate with the solubility matter results (Table 4.1). The enzyme probably occupied the free spaces of the matrix. It provided additional intermolecular interactions between starch and CMC and the other additives, resulting in a more concise, flexible, and resistant material. No significant difference ($p < 0.05$) for P5 film was observed when compared with P0 film (Fig. 4.4), which can be related to the papain homogeneity or presence of saturated aggregates along with the films. In general, all films presented great flexibility and could be used as beauty skin masks, being able to mold themselves to the surface of the face. In addition, the films did not disrupt easily and were strong enough to handle the external strength applied during the use of the mask or the transport and manipulation.

Figure 4.4: Tensile strength and elongation at break of the films containing the final papain concentrations P0 (0%), P1 (1%), P2 (2%), and P5 (5%) (w/w g of papain per g of total polymer in the films after the mixture).



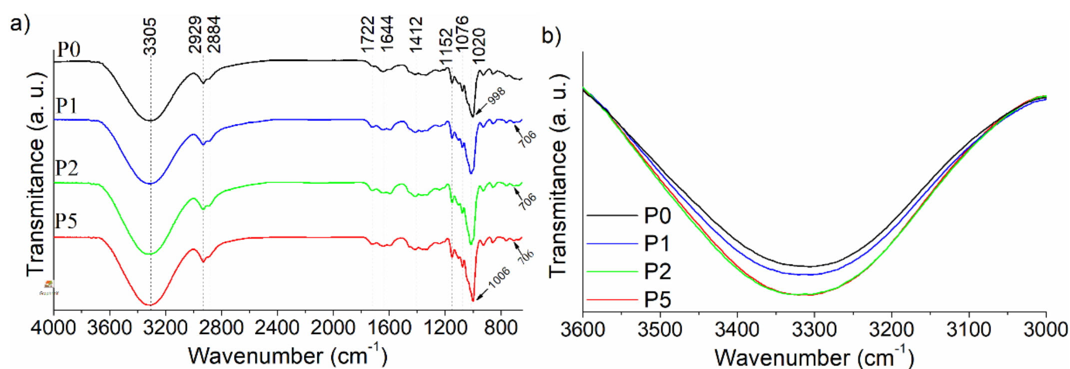
All values were expressed as mean ± standard error. Means within the same parameter having different letters are significantly different at the level of 5% ($p < 0.05$).

Figure 4.5 shows the FTIR spectrum of the films. The band near 3305 cm^{-1} was related to the stretching vibrations of ─OH groups and ─NH bond, the last being exclusive in the films with contained the enzyme. Probably, the superposition of both ─OH and ─NH peaks occurred when comparing P0 films to the other formulation (Fig. 5b), indicating the effect of papain incorporation on the polymeric matrixes. Moreover, it is known that strong hydrogen bonding can affect this region which can be related to possible intermolecular interactions that occurred in the films with papain and the other components. Similarly, an increase in the band intensity was observed in other studies that added papain into polymeric films and fibers (SHOBA et al., 2014; SILVA et al., 2016).

The incorporation of papain also changed the peak of 992 cm^{-1} to 1020 cm^{-1} of P0 and P1, and P2 films and to 1006 cm^{-1} for P5 film (Fig. 4.5a). This peak is attributed to ─CO stretching vibration and also to C─S stretching, the last bond found in the enzyme. Other studies previously reported the peak shifting with papain incorporation in polymeric materials (DUTRA et al., 2017; SHARMA et al., 2011). The peak of 705 cm^{-1} appeared in films P1, P2, and P5 and referred to the sulfide and disulfide bonds present in papain (SHOBA et al., 2014). The peak around 1644 cm^{-1} can be related to the ─OH bond and the characteristic peak of amide I present in proteins (SILVA et al., 2016). The remaining peaks (Figure 4.5a) were observed in films of starch and CMC. The peaks around 2880 – 2930 cm^{-1} are attributed to ─CH stretching, and the carboxylic ions (COO−) symmetric stretching was observed at 1412 cm^{-1}. Stretching vibrations of ─CO,

C—O—C (related to polysaccharide structure), and —OH (characteristic of water binding in materials) were observed at 1152 cm^{-1}, at 1076 cm^{-1}, and at 1723 cm^{-1}, respectively (SURIYATEM; AURAS; RACHTANAPUN, 2019; TAVARES et al., 2019; TONGDEESOONTORN et al., 2011; VERONESE et al., 2018).

Figure 4.5: Attenuated total reflectance Fourier transform infrared (ATR-FTIR) spectra of the films containing the final papain concentrations P0 (0%), P1 (1%), P2 (2%), and P5 (5%) (w/w g of papain per g of total polymer in the films after the mixture).



From the bands and peaks observed in Figure 4.5, starch, CMC, and the other components did not present groups that can lead to covalent immobilization of papain. In this sense, it is hypothesized that electrostatic interactions between the hydroxyl groups present in the film and the amino groups of the enzyme immobilize papain.

4.3.4 Proteolytic and exfoliating activity of the films

It is important to ensure that the active compound maintains its desired activity after processing the material. Table 4.2 presents the enzymatic activity of the films. It is interesting to mention that P0 film did not present any activity, which indicates that both polymers and the other additives (glycerol and citric acid) did not affect the substrates

All films presented proteolytic activity against casein and APSS substrate (Table 4.2), demonstrating that the films can hydrolyze soluble and non-soluble proteins. Concerning APSS hydrolysis, P5 film presented higher ($p > 0.05$) activity when compared to P1 and P2 films. These results agree with the previous results that indicate P1 and P2 film presents a more concise matrix than P5 film, which probably facilitated the papain release. On the other hand, the increase of papain significantly ($p > 0.05$) increased the

casein activity, which probably occurred due to the easy degradation of casein compared to APSS.

Table 4.2: Enzymatic activity of the films using casein and azo porcine skin substrate (APSS).

Film	Casein ($U_{casein}/g_{support}$)	APSS ($U_{APSS}/g_{support}$)
P1	0.3 ± 0.1 ^{c}	18.1 ± 2.2 ^{b}
P2	0.8 ± 0.1 ^{b}	22.2 ± 5.0 ^{b}
P5	1.7 ± 0.1 ^{a}	33.0 ± 4.9 ^{a}

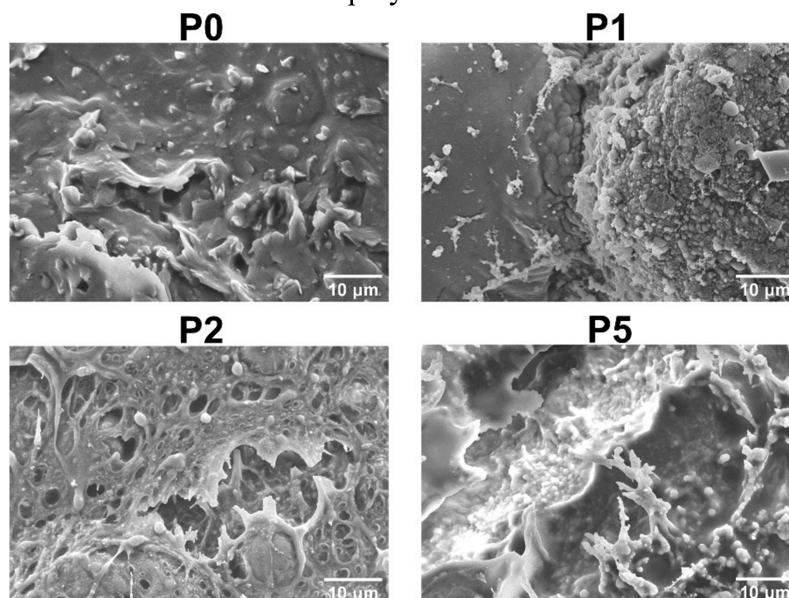
All values were expressed as mean ± standard error. According to the Tukey test, averages with the same subscript letter in the same column had no significant differences ($p < 0.05$).

The results show that P1, P2, and P5 films have proteolytic activity (Table 4.2), possibly being able to present the active property of exfoliants as beauty masks. To ensure that the films can exfoliate the skin, Figure 4.6 shows micrographs of porcine skin sections after the treatment of the films.

The control film (P0) appears not to have affected the skin structure after its application (Fig. 4.6). The skin maintained its structure, showing no pores or flaky structures. The micrograph of the P1 application seems to be more heterogenous than the P0 one, which can indicate some exfoliation by the presence of some raised. But in other regions, no evident exfoliation signals could be observed. On the other hand, both micrographs of the skin after the P2 and P5 film treatment showed fibrous and detached arrangements. Moreover, some “openings” could be observed along with the matrices of both P2 and P5 films, which indicate the action of the enzyme. These results indicate that the papain was able to promote the hydrolysis of the skin's proteins, having the desired exfoliating action.

A real procedure of a skincare routine with face masks longs around 20 min but varies for each individual and product. The measurement of enzyme activity with the substrates and the exfoliation of the skin was performed aiming at a real application of beauty face masks. From the results observed, it can be indicated that the masks present proteolytic activity during 30 min application, indicating that, at least after 30 min of use, the mask allowed the enzyme action. It is important to highlight that enzymes are pH and temperature dependents and that adding warm water and using solutions that can increase the skin's pH could help enzymatic exfoliation (TREVISOL et al., 2022). Additional studies about safety and periodicity must be performed to fully elucidate this topic.

Figure 4.6: Scanning electron micrographs of porcine skin sections after treatment of films containing 0 (P0), 1 (P1), 2 (P2), and 5% (P5) (w/w) g of papain per g of total polymer.



4.3.5 Stability

The stability test guides a cosmetic formulation for product safety and gives insights into the shelf-life of the product/material. Figure 4.7 demonstrates the accelerated stability test of the films and 0.5 g L^{-1} papain solution. The results indicate that the solution of free enzyme presented lower storage stability, and the films provided prolonged activity maintenance. Independently of the storage condition, at 30 days, papain in free form did not maintain its activity. The loss of enzymatic activity could be attributed to conformational changes in the enzyme and self-digestion. On the other hand, the polymeric matrix probably had difficulted with the conformational changes of papain. It inhibited the self-digestion of the enzyme by steric hindrance in the results of P1, P2, and P5 films (MIYAMOTO; WATANABE; ISHIHARA, 2004). Similar stability results of native papain were observed in the literature (MIYAMOTO; WATANABE; ISHIHARA, 2004; MOREIRA FILHO et al., 2020; SIM et al., 2000; VERDUZCO et al., 2021).

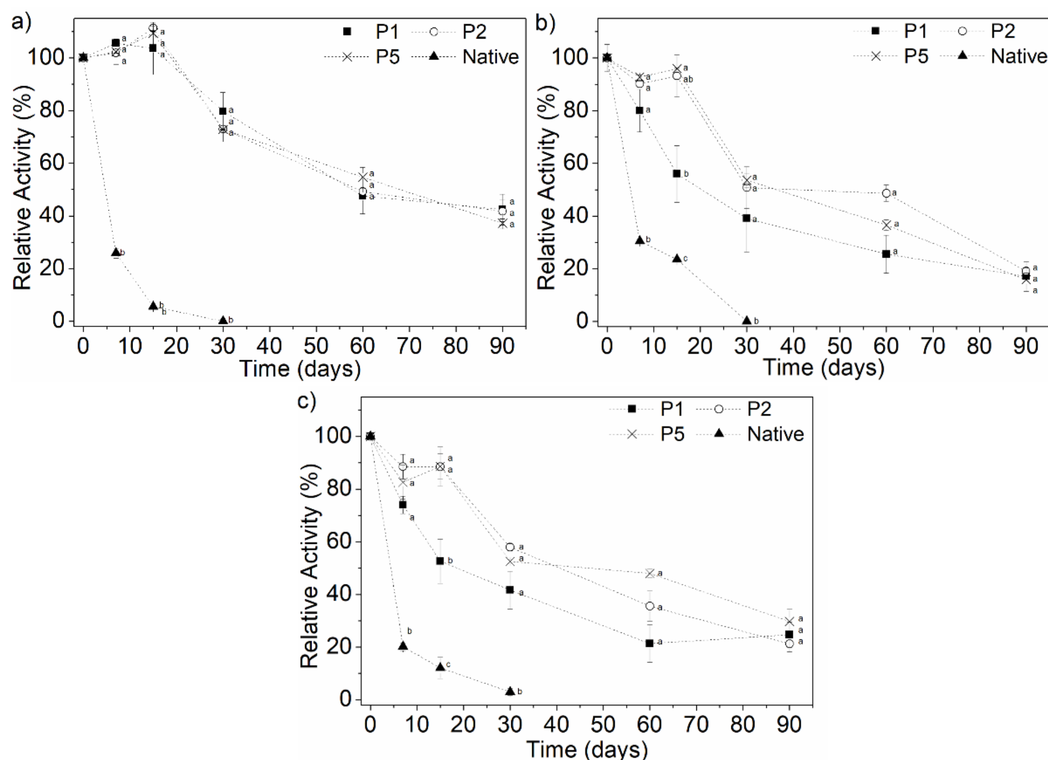
No activity reduction was observed for all formulations of the films until 15 days of storage ($p < 0.05$) under refrigeration conditions (Fig. 4.7a). After 30 days, the films presented nearly 70% of activity, reaching nearly 50% and 40% on days 60 and 90, respectively. No significant reduction was observed among all films at the same time of

storage ($p < 0.05$). Under room temperature (Fig. 4.7b), the films presented higher activity loss after 90 days, remaining only 20% of activity. P1 film had a fast activity decrease during this storage condition, reaching less than 60% of the remaining activity after 15 days of storage. This result was significantly different ($p > 0.05$) from P5; the statistics analysis demonstrates that no difference occurred between P2 and P5 films and P1 and P2 formulations. At 37 °C (Fig. 4.7c), the films presented a similar pattern to room temperature: after 15 days, P1 film had less ($p < 0.05$) activity (reaching nearly 60%) than P2 and P5 films, which maintained near 90% of papain activity. After 30 days, the activity reduction rate was similar for all films ($p < 0.05$). On day 90, less than 40% of activity was observed among the films.

Few literature reports have described the stability of papain in cosmetic formulations. For example, Pinto et al. (2011) tested diverse emulsions containing papain and a modified papain form (methoxypolythylene glycol succinimidylsuccinate papain). The modified papain provided the best stability for 90 days. The results of the non-modified papain demonstrate a rapid decrease in activity. Similar to the present work, the refrigeration condition was the best condition for storing the enzyme. However, almost no activity remained at 40 °C after 90 days. Sim et al. (2000) conjugated papain with polymeric matrices and observed that 1,3-glucan from *Schizophyllum commune* provided a better protective effect and maintained almost 100% of activity for 30 days. On the other hand, these authors observed that dextran or polyethylene glycol conjugation provided less than 50% of the remaining activity. No difference was observed in the storage at 25 and 45 °C with glucan.

The refrigerated (Fig. 4.7a) condition resulted in a slower pattern of activity lost than the room (Fig. 4.7b) or 37 °C (Fig. 4.7c) environments. After 90 days, no significant difference ($p < 0.05$) was observed in enzyme activity among the films for each temperature, demonstrating that the papain concentration did not affect this parameter. On the other hand, more than 40% of activity remained for the refrigerated storage against low amounts for the other conditions. These results can be attributed to various factors such as no light exposure; low microbial activity due to low temperature and low humidity; distance from the optimum temperature of papain, resulting in low possible autolysis reactions; reduction of the degradation rate of all or part of the compounds due to temperature, etc. (MOREIRA FILHO et al., 2020; PINTO et al., 2011; VERDUZCO et al., 2021).

Figure 4.7: Papain activity variation of the films and free form during accelerated stability test under the conditions of 6 ± 2 °C (a), 22 ± 5 °C (b), and 37 ± 3 °C (c). P1, P2, and P5 correspond to films containing 1, 2, and 5% (w/w) g of papain per g of total polymer in the films after the mixture), respectively. Native corresponds to 0.5 g L-1 papain solution. Symbols with the same letter indicate that no significant difference ($p < 0.05$) was observed for that period.



During the stability test, no noteworthy organoleptic changes were observed from the films (data not shown). The films at refrigerated condition presented some mass variation in the first 7 days, probably related to a less moist environment, possibly resulting in water loss. The stability test results demonstrated that the refrigeration condition is the best environment to keep the films of starch and CMC with papain incorporation to be used as beauty masks, with more than 70 and 40% of activity remaining at day 30 and 90, respectively. It is important to highlight that no modification of papain was performed and no use of preservatives in the formulation, which could increase the resistance of papain activity loss.

4.4 CONCLUSION

Papain was incorporated into films of starch and CMC, and the obtained material was demonstrated to maintain the enzyme's activity. The immobilization provided better

activity maintenance during storage compared with the free enzyme. The incorporation of papain modified the physico-chemical and morphological properties of the films. The increase in papain concentration generally provided more flexibility and solubility resistance. The results demonstrated that the films have remarkable characteristics and can be potentially used as beauty masks with enzymatic exfoliation activity.

Both P2 and P5 presented similar exfoliation activity (Fig. 4.6) and maintenance of the activity of papain during storage (Fig. 4.7). However, taking into account the physico-chemical properties it seems that P2 film presented better results (mainly in mechanical properties, Fig 4.3) while P5 presented higher enzyme activity (Table 4.3). The choice between both formulations depends on the enzyme activity needed and the region in which the product is being used, but it seems that the P2 formulation is the "ideal" choice.

CHAPTER 5: DIFFERENT ADSORPTION STRATEGIES FOR PAPAIN IMMOBILIZATION IN BIOPOLYMERIC-BASED COSMETIC SHEET MASKS

This chapter contains a manuscript that was not submitted to any journal.

5.1 INTRODUCTION

Cosmetics have the importance of improving the appearance and health of the skin and, consequently, promoting the psychosocial-biological match of the self-image of humans (MORGANTI et al., 2020; RATTAN, 2015). Among the cosmetics, beauty face masks are known for their effectiveness, accessibility, ease, and quick use (PERUGINI et al., 2019) and are used to supplement the daily skincare routine (MORGANTI et al., 2020). The face masks can contain several ingredients, such as exfoliants, vitamins, moisturizers, brightening ingredients, herbals, proteins, etc., which makes this type of cosmetic very versatile. Depending on the application method, beauty face masks can be classified as a sheet, peel-off, or rinse-off masks (NILFOROUSHZADEH et al., 2018).

Sheet face masks are made of thin paper or polymeric materials that can be incorporated or soaked with different ingredients to achieve desired characteristics. Due to the prevention of water evaporation, this face mask is known to improve skin hydration and the penetration of ingredients (PERUGINI et al., 2019). Commercially available beauty face masks were generally produced with fossil-based materials due to low costs and processing. However, due to environmental problems, face masks have single-use before disposal, and the use of biopolymers was stimulated to aim for the production of both eco- and skin-friendly materials (MORGANTI et al., 2020). Moreover, this cosmetic requires high hydrophilicity, water absorption, non-toxicity, and sufficient mechanical properties to cover the face that can be easily achieved using biopolymers (ZHAO et al., 2019).

Starch is a low-cost polysaccharide obtained from plants with good film-forming properties, making it an interesting biodegradable polymer for producing face masks (COLTELLI et al., 2018; JHA et al., 2020). However, starch-based films have low mechanical and barrier properties (JHA et al., 2020; SURIYATEM; AURAS; RACHTANAPUN, 2019), which may provide undesired properties for this application. Carboxymethyl cellulose (CMC) is a polysaccharide derived from cellulose that has

interesting properties to be applied as beauty masks, such as excellent water absorption, flexibility, and barrier properties (GAO et al., 2017; GHORPADE; YADAV; DIAS, 2017; TREVISOL et al., 2020). Films of the blend of both polymers have already been evaluated as food packaging or edible material ((ANTOSIK; WILPISZEWSKA, 2018; GHANBARZADEH; ALMASI; ENTEZAMI, 2010; JHA et al., 2020; MA; CHANG; YU, 2008; PUTRI; SETIAWAN; ANGGRAINI, 2017; SURIYATEM; AURAS; RACHTANAPUN, 2019; TAVARES et al., 2019; WAHYUNINGTYAS; DINATA, 2018) and showed that some undesired properties of starch films were reduced when the polymer is blended with CMC (SURIYATEM; AURAS; RACHTANAPUN, 2019).

Papain (EC 3.4.22.2) is a proteolytic enzyme extracted from the latex of papaya (*Carica papaya*). This cysteine endopeptidase leads the industrial sales among other plant or microbial proteases (FERNÁNDEZ-LUCAS; CASTAÑEDA; HORMIGO, 2017). Papain has been used in cosmetic and pharmaceutical products as an exfoliant, drug penetration enhancer, and wound debridement agent due to the proteolytic action of the enzyme in the human skin proteins (Chapter 3). Several studies have researched the immobilization of papain in polymeric films or membranes (ASANARONG et al., 2021; DUTRA et al., 2017; MOREIRA FILHO et al., 2020; SANTOS et al., 2021; VASCONCELOS et al., 2020; WONGPHAN et al., 2022), which can extrapolate to using this enzyme in beauty mask materials.

In our previous work (Chapter 4), it was evaluated the incorporation of different amounts of papain in starch- and CMC-based films aiming for cosmetic application. Despite producing films with proteolytic activity and good enzyme distribution, part of the enzyme was not accessible to the substrate due to mass transfer difficulty. The present work aimed to use different strategies to immobilize the papain on the surface of masks to produce cosmetics with exfoliant properties. Films made of the blend of starch and CMC were used as support. Methodologies that involve toxic/irritant chemicals to immobilize were not used to prevent the production of possible non-skin safety masks.

5.2 MATERIAL AND METHODS

5.2.1 Material

The films were composed of medium-viscosity sodium carboxymethyl cellulose (degree of substitution 0.6, Neon Química, Brazil), potato starch (Shambala Naturais,

Brazil), glycerol (Sigma-Aldrich Chemical, USA), and citric acid (Neon Química, Brazil). Papain (from *Carica papaya*, activity ≥ 3.6 units/mg of protein, 23.4 kDa, Sigma-Aldrich Chemical, US) was immobilized in the films. The proteolytic activity was determined with sodium casein (Dinâmica Química, Brazil), L-Cysteine hydrochloride (Vetec, Brazil), tyrosine (Synth, Brazil), and trichloroacetic acid (TCA, Neon, Brazil). All the reagents were of analytical-grade quality.

5.2.2 Preparation of starch- and CMC-based films and papain immobilization

The films were prepared similarly to Chapter 4. Briefly, 1:1 mass ratio of 5% (w/v) of starch solution and 1% (w/v) of CMC solution were mixed; 1% (w/v) of glycerol was added to the mixture. The filmogenic solution was stirred by mechanical stirring (RW 20, IKA, GE) at 900 rpm for 30 min at room temperature. Before the mixture, the starch solution containing 0.5% (w/v) of citric acid was heated to 80 °C for 30 minutes and cooled to room temperature. The filmogenic solution was poured into polystyrene dishes (0.56 g of filmogenic solution per cm^2) and dried in a fume hood for 36 h at room temperature.

The immobilization of papain was performed by adsorbing the enzyme on the surface of the films according to the method described by Castro et al. (2022) and Simões et al. (2021), with some modifications. The obtained films (d = 9 cm) were not detached from their molds, and 4.6 mL of 1% (w/v) papain solution was added to the top of the films. The papain solution was prepared in 100 mM of phosphate buffer solution. The immobilization evaluated three different pHs of the enzyme solution: 6.0, 7.0, and 8.0. Then, the films were dried in a convective oven at 30 °C for 4 h, approximately.

The effects of previous acid hydrolysis of the surface of the films before adding the enzyme solution were also evaluated (HANUŠOVÁ et al., 2013; SHIM et al., 2017). In this case, 4.6 mL of HCl solution, varying the concentration from 0.05 to 0.20% (w/v), was added to each dried film (d = 9 cm). The films were dried for 3 h at 30 °C in a convective oven. After the first drying, 4.6 mL of 1% (w/v) papain solution was added to the surface of the films, and the films were dried again using the same conditions as before. The addition of distilled water or buffer solution previously the papain addition was also evaluated.

5.2.2.1 Plasma treatment

The plasma treatment was performed by a barrier dielectric discharge (DBD) reactor with two stainless steel electrodes and was performed as described Heidemann et al. (2019), with some modifications.. The reactor was coupled with a high voltage source (0130, Inergiae, Brazil), and the reaction was performed at atmospheric pressure using air as ionizing gas. The dried starch- and CMC-based films were not detached from the Petri dishes to prevent the formation of raised surfaces. The supports containing the films were placed in the center of the lower electrode, and the plasma was discharged for 2 min. The plasma discharge parameters were set at 120 Hz and 23 kV and the distance between the electrode and film was set to 2 mm, according to previous tests.

After the treatment, 4.6 mL of 1% (w/v) of papain solution at different pH, 6.0, 7.0, and 8.0, in phosphate buffer (100 mM), was dipped on the surface of the films. In addition, some tests were performed maintaining the films for 30 min before the enzyme immobilization. Then, the films were dried at 30 °C for 3 h (section 5.2.2). The proteolytic activity of the films was performed according to section 5.2.3. A control sample was performed by treating the Petri dishes without any film and dipping the papain solution after the treatment to evaluate the enzyme activity.

5.2.3 Proteolytic activity determination

The proteolytic activity of the films was determined using casein as a substrate. The films were maintained in their supports, and 2 mL of phosphate buffer solution (100 mM, pH 7.5) was added. Then, 4.6 mL of 0.65% (w/v) casein with a solution of 3 mM of L-Cysteine hydrochloride was added, and the dishes were incubated in an orbital shaker (TE-424, TECNAL, Brazil) at 37 °C and 70 rpm for 10 min. The reaction was stopped by removing the films and adding 4.6 mL of 10% (w/v) TCA solution. The negative controls were performed by adding the TCA solution before the casein. Then, the solution was centrifuged for 10 min at 8,170 xg, and the absorbance of the supernatant was observed spectrophotometrically (U-2900, Hitachi, Japan) at 280 nm. The obtained results were correlated with a previously performed tyrosine standard curve. One unit of caseinolytic activity (U) was defined as the amount of enzyme required to liberate 1 μmol of tyrosine per minute under the defined assay conditions.

Since the enzyme can endure conformational changes and be immobilized in regions which can be difficult to mass transfer, the recovered activity (RA) was determined. RA percentage was defined as the ratio of the activity of the immobilized enzyme on the support per the enzymatic activity of the free enzyme, as shown in Eq. 5.1.

$$RA = \left(\frac{Activity\ of\ the\ immobilized\ papain}{Activity\ of\ the\ free\ enzyme}\right) x\ 100 \tag{5.1}$$

In addition, the ratio of the immobilized activity was divided by the area of the support used. Immobilized activity (IA) was determined according to Eq. 5.2.

$$IA = \left(\frac{Activity\ of\ the\ immobilized\ papain}{Area\ of\ the\ immobilization\ support}\right) \tag{5.2}$$

5.2.3.1 Determination of Michaelis-Menten kinetic parameters

The catalytic activities of free and immobilized enzymes were investigated at different casein concentrations: 0.05 to 1.25% (w/v). The maximum velocity (V_{max}) and the Michaelis constant (K_M) were obtained from Michaelis-Menten plots. The turnover number (k_{cat}) was calculated by dividing v_{max} per total of enzyme that was used in the assay. The experiments were conducted at 34 °C and pH 7.5 (phosphate buffer solution, 100 mM).

5.2.4 Physic-chemical characterization of the films

5.2.4.1 Thickness and mechanical properties

The thickness of the films was measured using a digital micrometer (MDC-25P, Mitutoyo, Japan). For each film (n = 8), an average thickness of 5 random positions of each film was determined. The presence of any visible defects caused the discard of the specimen.

The mechanical properties of tensile strength and elongation at break were determined in a texture meter (TA.HD.plus Texture Analyzer, Stable Micro Systems UK) in accordance with ASTM standard method D88-02 (ASTM D88-02, 2002). A 50 N load

cell capacity was used, and the tests were performed using 1 mm/s of crosshead speed and 50 mm of initial grip spacing. Five samples for each film were used.

5.2.4.2 Water-behavior properties

The films were air dried at 105 °C for 24 h to determine their moisture content (MC). MC was expressed as the percentage of g of water per 100 g of wet material. The solubility matter (SM) of the films was also determined by gravimetric method, in quadruplicate. Briefly, each film was weighed (W_i) and then immersed in 50 mL of distilled water. After maintaining the at 30 °C for 24 h, the excess water was removed, and they were weighed (W_f). SM was determined according to Eq. 5.3.

$$SM = \left(\frac{W_i - W_f}{W_i}\right) x\ 100 \tag{5.3}$$

The water contact angle was determined using sessile drop method with a goniometer (Ramé-Hartz 250, Ramé-Hartz Instrument Co., Germany). Films (n = 4, 1 cm x 2.5 cm) were fixed on glass slides, and the contact angle of one drop (50 µL) of distilled water released over the surface was recorded. Five measurements were performed for each film.

The water vapor permeability (WVP) was gravimetrically evaluated for each film (n = 3). The experiments were conducted at room temperature in accordance with ASTM E96/E96M (ASTM E96 / E96M-10, 2010). Films were sealed in oval aluminum capsules containing silica gel; the capsules had a permeation area (A) of 0.0031 m^2. Then, the capsules were kept at 25 °C in a glass container and controlled relative humidity of 75%. The capsules containing the films were weighed every 2 h (for 24 h total), and the permeation rate (G, $g/(m^2.h)$) was obtained by the slope of linear regression of weight versus time. The WVP was calculated, as shown in Eq. 5.4, where L is the thickness of each film (mm), P is the saturation vapor pressure of water (Pa) at the test temperature (25°C), and ΔRH was the difference of relative humidity of the glass container (0.755) and the inner of the capsule (0.02).

$$WVP = \frac{(G \cdot L)}{(A \cdot P \cdot \Delta RH)} \tag{5.4}$$

5.2.4.3 Chemical composition determination

Fourier Transform Infrared attenuated total reflectance (FTIR-ATR) spectroscopy of the films was obtained with a spectrometer (Cary 600, Agilent, US) to evaluate the chemical bonds of the films and components. The tests were performed between 650 and 4000 cm^{-1} with 32 scans at 4 cm^{-1} resolution.

5.2.4.4 Thermal properties

The thermal transitions of the films and their components were analyzed in a differential scanning calorimeter (PerkinElmer Jade, USA). The samples (10 mg) were sealed in hermetical aluminum pans and maintained at room temperature for 1 h to equilibrate. Then, the samples were heated from 20 to 200 °C under an N_2 flow rate of 50 mL/min. A heating rate of 5°C/min was used. An empty aluminum pan was used as a reference. The starch powder sample was previously mixed with distilled water (1:2 w/w starch: water ratio) to enable the occurrence of the gelatinization process, as previously performed by Alves et al. (2021). The thermal transitions were obtained from the differential scanning calorimetry (DSC) thermograms with Pyris Data Analysis software.

5.2.5 Statistical analysis

Analysis of variance (ANOVA) and Tukey test was used to statistically determine the significant differences ($p < 0.05$) among averages using the software STATISTICA (version 7.0). The coefficient of determination (R^2) statistical index was used to evaluate the performance of the models of Michaelis-Menten kinetics.

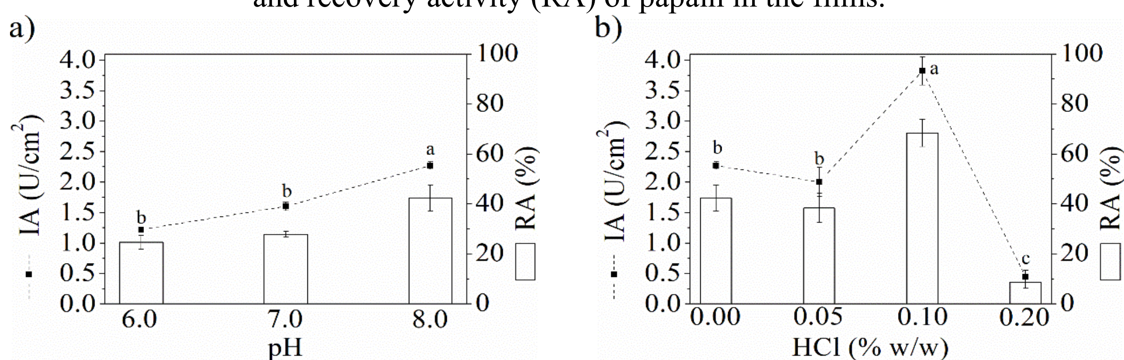
5.3 REULTS AND DISCUSSION

5.3.1 Effect of the pH and acid activation in the immobilization process

Figure 5.1 summarizes the results of IA and RA of papain for different treatments of starch- and CMC-based films. The results showed that the pH had significantly affected ($p > 0.05$) both IA and RA results. Using the buffer solution of pH 8.0 presented the best results for the immobilization of papain (Figure 5.1a). The use of

more acid buffers probably provided electrostatic repulsion effects between the charged groups of papain and the films. In addition, it is usually observed that the best adsorption of proteins occurs at their isoelectric points. The isoelectric point of papain is 8.75 (CAHYANINGRUM et al., 2013). The present results are similar to other studies that performed papain adsorption in supports (ALPAY; UYGUN, 2015; CAHYANINGRUM et al., 2013; CHEN et al., 2009). The best IA and RA results were 2.267 U/cm^2 and 42% in the pH 8.0 assay.

Figure 5.1: Effect of pH (a) and acid treatment (b) for the immobilization activity (IA) and recovery activity (RA) of papain in the films.



According to the Tukey test, averages with the same letter had no significant differences ($p < 0.05$).

The activation of the surface of the films was obtained with acid treatment. From Figure 5.1b, it can be observed that the use of 0.10% (w/w) of HCl significantly ($p > 0.05$) improved the IA and RA of the films, reaching 3.82 U/cm^2 and 68.4%, respectively. The augment of HCl to 0.2% (w/w) reduced ($p > 0.05$) both IA and RA, possibly due to the start of degradation of polymeric matrix or to the presence of acid characteristic that was not easily neutralized even after water and buffer treatment. Similarly, Hanušová et al. (2013) observed a reduction in enzyme immobilization when their polyamide and ionomer films were treated with higher HCl concentrations (from 3 to 6 M). No significant difference ($p < 0.05$) was observed using 0.05% (w/w) of HCl when compared with no acid treatment. This result indicates that no effective surface modification occurred.

The films were named papain film (PF) and acidified papain film (APF) with the immobilization of papain at pH 8.00 with none and acid surface treatment (0.10% HCl w/w), respectively.

5.3.1.1 Considerations of plasma treatment

The results of plasma treatment are shown in Table 5.1. Similarly, as observed in the pH studies in Section 5.3.1, the best immobilization condition occurred at pH 8.0. However, the results observed in Figure 5.1 show a reduction in recovery activity. The possible explanation occurs by the ozone formation on the surface of the films that inactivate the enzyme, as confirmed by the Control assay: ozone molecules are known to inactivate papain even at low concentrations (TODD, 1958). Even performing the immobilization after 30 min did not improve the results. The reaction seems irreversible even after drying the films for 3 h and waiting overnight for the realization of proteolytic activity measurement (data not shown). A possible manner to avoid the results could be achieved using other ionizing gas, such as nitrogen or ammonia, which were not tested in the present study.

Table 5.1: Results of different pH of papain solution after the plasma treatment on the surface of the films.

Treatment	Recovery activity (RA, %)
pH 6.0	7.7 ± 1.9
pH 7.0	13.9 ± 1.0
pH 8.0	20.2 ± 1.9
pH 8.0 after 30 min of waiting	25.8 ± 3.1
Control	0.0

5.3.1.2 Effect of immobilization of papain in the Michaelis-Menten parameters

There was an increase of K_M and a decrease of V_{max} when papain was immobilized in the PF films, as indicated in Table 5.2. This variance indicates a loss of papain activity after the immobilization procedure (METIN; ALVER, 2016). Steric hindrance, mass transfer limitation, and/or loss of the necessary enzyme flexibility for the formation of enzyme-substrate complex probably are the main causes of the changes in the kinetic parameters when the enzyme was immobilized (HOMAEI; SAMARI, 2017). These changes of k_M and v_{max} have been reported for papain (GANAPATHI-DESAI; BUTTERFIELD; BHATTACHARYYA, 1998; HOMAEI, 2015; HOMAEI; SAMARI, 2017; ZHANG et al., 2016). However, when comparing native and APF results no significant difference was observed ($p < 0.05$).

Table 5.2: Michaelis-Menten kinetic parameters for free and immobilized papain.

Papain	Michaelis-Menten Parameters			
	k_M (μM)	v_{max} (μM/min)	k_{cat} (1/min)	R^2
Native	193.41 ± 18.55 [a]	181.58 ± 8.52 [a]	0.42 ± 0.06	0.958
Papain film (PF)	299.25 ± 38.09 [b]	91.74 ± 10.51 [b]	0.21 ± 0.05	0.869
Acidified papain film (APF)	234.54 ± 29.45 [ab]	155.97 ± 14.22 [ab]	0.36 ± 0.08	0.914

Native corresponds to 1% (w/v) of papain solution in phosphate buffer (100 mM, pH 7.5); PF corresponds to film treated with papain solution at pH 8.00; APF corresponds to film treated with 0.10% (wt) of HCl solution followed by treatment with papain solution at pH 8.00. According to the Tukey test, averages with the same superscript letter in the same line had no significant differences ($p < 0.05$).

The immobilization of papain in APF provided better results than PF (lower k_M and higher v_{max}, Table 5.2). This result is directly correlated to IA and RA values from APF assay (Figure 5.1b), i.e., there was a higher amount of papain to promote the proteolysis. Moreover, the Michaelis-Menten model has limitations regarding using non-soluble immobilized enzymes: lower R^2 values were observed for PF and APF assays.

There was a decrease of k_{cat} for PF and APF assays when comparing the native papain (Table 5.2). The reduction of this parameter occurs because there is a mass transfer restriction for the substrate to find the active site of the enzyme due to conformational restrictions (BAIDAMSHINA et al., 2021; HOMAEI, 2015). Similarly, there was a reduction of specificity constant (k_{cat}/k_M) from 0.0022 to 0.0007 and 0.0015 1/(min.μM) for native and PF and APF assays, respectively. Nevertheless, the acidification of the films provided better immobilization, corresponding to APF formulation.

5.3.2 Physic-chemical characterization of the films

5.3.2.1 Thickness and water-behavior properties of the films

In addition, to PF and APF formulations, control film (CF) and acidified film (AF) were also produced without papain immobilization.

The thickness of the films (Table 5.3) was not affected ($p < 0.05$) by the acid activation or papain immobilization. These results can be correlated to the low amount of both components that were added to PF, AF, and APF formulations, which were insufficient to increase the thickness. Moreover, the results also indicate that there was

no electrostatic repulsion between the components due to no alteration of film density. Regarding the application of the films as beauty masks, there is a tendency to use thin masks below the human skin (which varies from 0.5 to 2 mm), indicating that the obtained masks are feasible for this type of use.

Table 5.3: Thickness, moisture content (MC), solubility matter (SM), water absorption (WA), water contact angle (WCA), and water vapor permeability (WVP) of the films.

Characterization	Film			
	Control film (CF)	Papain film (PF)	Acidified film (AF)	Acidified papain film (APF)
Thickness (μm)	202 ± 40^a	199 ± 36^a	201 ± 40^a	192 ± 10^a
MC (%)	50.3 ± 1.1^a	49.8 ± 1.0^a	51.3 ± 1.2^a	52.2 ± 4.8^a
SM (%)	32.6 ± 6.8^a	32.4 ± 7.6^a	38.2 ± 5.7^a	33.4 ± 4.8^a
WA (g/g)	1.85 ± 0.32^b	1.34 ± 0.19^a	1.78 ± 0.20^b	1.18 ± 0.11^a
WCA (°)	37.5 ± 4.0^a	58.9 ± 9.0^b	40.6 ± 2.3^a	52.4 ± 7.5^b
WVP (x 10^{-4} (g.mm)/(m2.h.Pa))	6.03 ± 0.63^a	5.36 ± 0.19^a	5.67 ± 0.87^a	5.64 ± 0.30^a

CF corresponds to film without acid or papain treatment; PF corresponds to film treated with papain solution at pH 8.00; AF corresponds to film treated with 0.10% (wt) of HCl solution; APF corresponds to film treated with 0.10% (w/w) of HCl solution followed by treatment with papain solution at pH 8.00. According to the Tukey test, averages with the same superscript letter in the same line had no significant differences ($p < 0.05$).

The immobilization of papain and/or the activation step did not influence ($p < 0.05$) the MC and SM of the films, as shown in Table 5.3. These results can be correlated with the characteristics of the polymeric matrix (polymers, crosslinking, and plasticizer) than the papain and HCl. Interestingly, the acidification did not damage or facilitate the hydrolytic degradation of the films, indicating that the concentration of 0.10% (w/w) of HCl preserved the desired properties of the film and improved the papain immobilization. All films presented similar SM results to other starch- and CMC-based films (GHANBARZADEH; ALMASI; ENTEZAMI, 2010; JHA et al., 2020; PUTRI; SETIAWAN; ANGGRAINI, 2017). Compared with the films from Chapter 4, the films in the present study presented more MC and SM properties. The films presented more water content due to adding water solutions to immobilize the enzyme. The SM results can be correlated to the fact that papain did not have structural properties in the present study and the increase of glycerol content which may help the water solubilization of the films.

The addition of papain modified the WA and WCA of the films (Table 5.3). The results were significantly ($p > 0.05$) reduced for WA and improved for WCA in PF and APF formulations compared to CF and AF. These changes can be explained by the presence of hydrophobic amino acid residues in papain, which lead to hydrophobic characteristics and, consequently, reduce the water that could be bound to the films. In their study, Wongphan et al. (2022)also observed an increase in WCA values when papain was incorporated into their starch films. Still, the authors explained that papain reduced the water dissolution and led to polymer aggregation, enhancing the hydrophobicity of the surface of the material. The reduction of FA was also observed in other studies when papain was immobilized in polymeric matrices (ASANARONG et al., 2021; DUTRA et al., 2017).

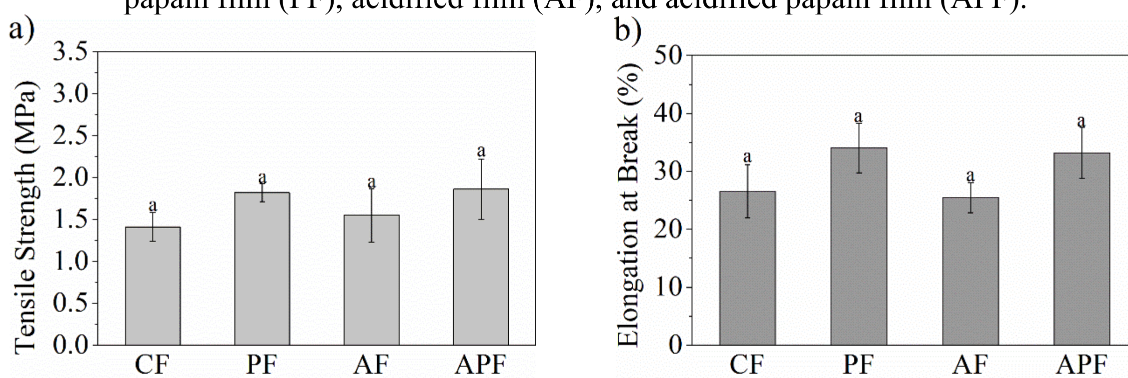
In evaluating the WVP through a beauty mask, it is important to allow skin respiration when using the material (AFONSO et al., 2019). At the same time, maintain the environment sufficiently moist to the action of the active ingredient. Table 5.3 shows the results of WVP of the films. The acid activation did not ($p < 0.05$) affect the results (AF and APF formulations). This result is interesting to ensure that the use of HCl does not sufficiently damage the structure of the films, corroborating with SM result, and indicating that the films can be used as beauty masks without losing their structure. The WVP of the films was similar to those cited in other studies of films of starch and CMC (GHANBARZADEH; ALMASI; ENTEZAMI, 2010; JHA et al., 2020; MA; CHANG; YU, 2008; SURIYATEM; AURAS; RACHTANAPUN, 2019; TAVARES et al., 2019).

Similarly, the papain immobilization did not ($p < 0.05$) influence the WVP results of the films (Table 5.3, PF and APF films). Even though papain has a certain hydrophobic property, its presence did not difficulted the vapor water passage across the matrix. The results suggest that water mass transfer is majorly affected by the polymeric matrix itself, and the hydrophobic regions containing papain were insignificant in affecting water diffusion (WONGPHAN et al., 2022). The incorporation of papain did not influence the results of water vapor diffusion, at low enzyme concentrations, for starch- (WONGPHAN et al., 2022). or chitosan-based films (SANTOS et al., 2021), for example.

5.3.2.2 Mechanical properties of the films

The results of the mechanical properties of the films are shown in Figure 5.2. Regarding the tensile strength, Figure 5.2a demonstrates that no significant difference ($p < 0.05$) was observed among the formulations. These results suggest that both papain immobilization (PF and APF) and/or acid activation (AF and APF) did not affect the control sample (CF). Similarly, no significant difference ($p < 0.05$) was observed in elongation at break results (Figure 5.2b) for all formulations.

Figure 5.2: Tensile strength (a) and elongation at break (b) of the control film (CF), papain film (PF), acidified film (AF), and acidified papain film (APF).



CF corresponds to film without acid or papain treatment; PF corresponds to film treated with papain solution at pH 8.00; AF corresponds to film treated with 0.10% (w/w) of HCl solution; APF corresponds to film treated with 0.10% (w/w) of HCl solution followed by treatment with papain solution at pH 8.00. According to the Tukey test, averages with the same letter had no significant differences ($p < 0.05$).

Some studies have already reported that adding papain to polymeric films improves the mechanical properties of the materials. Still, high enzyme concentrations tend to lead to the reduction of this properties (ASANARONG et al., 2021; SANTOS et al., 2021; WONGPHAN et al., 2022). The concentration of papain that was immobilized in PF and APF formulations did not was sufficiently high to reduce both tensile strength and elongation at break (Figure 5.2a and Figure 5.2b, respectively). In addition, the immobilization by adsorption, differently by direct dissolution of the enzyme in the filmogenic mixture, did not lead the enzyme to present structural properties.

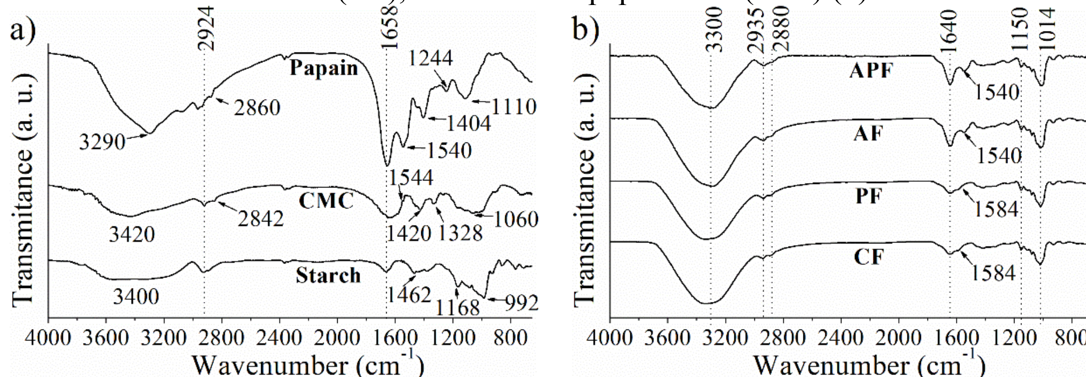
The present results of mechanical properties are close to other films with similar compositions (ANTOSIK; WILPISZEWSKA, 2018; MA; CHANG; YU, 2008; WAHYUNINGTYAS; DINATA, 2018). However, other studies described higher tensile strength values or elongation at break (GHANBARZADEH; ALMASI; ENTEZAMI,

2010; SURIYATEM; AURAS; RACHTANAPUN, 2019; TAVARES et al., 2019). This discrepancy can be explained by the difference in composition of the films and polymers. It is known that moisture content affects the mechanical properties (MA; CHANG; YU, 2008), which could explain the reduction of the tensile strength (Figure 5.2a) compared to the results of Chapter 4. Moreover, there was a reduction in plasticizer content (compared to Chapter 4) in the present study, which probably reduced the flexibility of the films (Figure 5.2b). From the perspective of applying the films as beauty masks, they seem to have sufficient resistance and flexibility to act on the skin (ZHAO et al., 2019).

5.3.2.3 Chemical composition determination

Figure 5.3a shows the FTIR spectrum of papain, CMC, and starch powders used to produce the active films. Regarding papain, both amide I and amide II characteristic peaks were observed at 1658 and 1540 cm^{-1}, respectively (ASANARONG et al., 2021). A prominent band related to ─NH stretching of a secondary N-substituted amide is shown at 3290 cm^{-1} (SHOBA et al., 2014). The weak peaks at 1404 and 1244 cm^{-1} are assigned to ─CH deformation of alkyl chains of the amino acids (DUTRA et al., 2017). The small band at 1110 cm^{-1} occurred due to the ─CS stretching of sulfide groups present in the enzyme (DUTRA et al., 2017; SHOBA et al., 2014). Symmetric and asymmetric stretching of CH_3 and CH_2 groups, the carbonic chains of polypeptides appeared at the bands of 2924 and 2860 cm^{-1}, respectively (DUTRA et al., 2017).

Figure 5.3: Fourier Transform Infrared (FTIR) spectroscopy of starch, carboxymethyl cellulose (CMC), papain powders (a) and control film (CF), papain film (PF), acidified film (AF), and acidified papain film (APF) (b).



CF corresponds to film without acid or papain treatment; PF corresponds to film treated with papain solution at pH 8.00; AF corresponds to film treated with 0.10% (w/w) of HCl solution; APF corresponds to film treated with 0.10% (w/w) of HCl solution followed by treatment with papain solution at pH 8.00.

In the analysis of CMC powder (Figure 5.3a), the peak at 1060 cm^{-1} was related to the C─O─C stretching vibration (GHORPADE; YADAV; DIAS, 2017), whereas the peaks at 1630 and 1420 and 1328 cm^{-1} to ─COOH asymmetric and symmetric stretching, respectively (CAPANEMA et al., 2018). The peak of 1540 cm^{-1} was also due to COO─ stretching vibrations (JHA et al., 2020). For starch, it was also observed ─CH stretching peak at 2924 cm^{-1} and ─OH and the ─CO stretching bonds of carbohydrates were seen at 1658 and 1168 cm^{-1}. The peak at 992 cm^{-1} is typical for partial crystalline materials and corresponds to intramolecular hydrogen bonding at the C─6 structure (ALVES et al., 2021; LOMELÍ-RAMÍREZ et al., 2014). Both polymers showed the hydroxyl groups band around 3400 cm^{-1}.

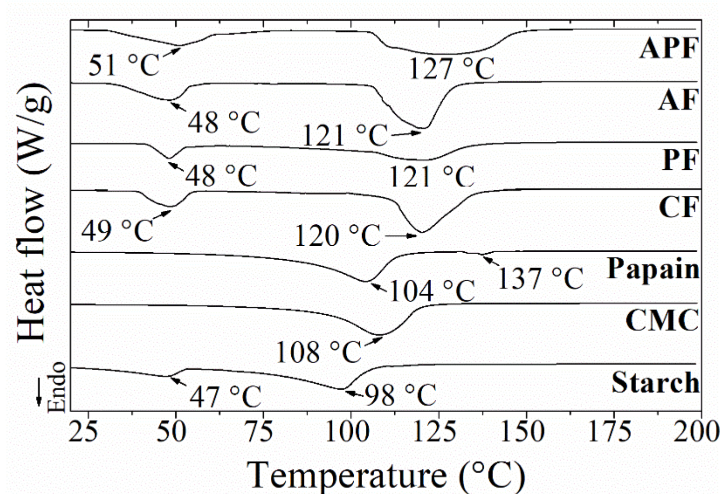
Figure 5.3b shows the spectrum of the films. All films generally presented the same peaks correlated to the polymers and papain. Differently, from observed in Chapter 4, there is no expressive increase in the band around 3700 – 3200 cm^{-1} when papain was immobilized. The AF film presented a higher increase in the band and probably is related to the presence of acid or the formation of hydroxyl groups due to the activation of the surface of the films. The peak at 1584 cm^{-1} from CF and PF formulations was displaced (and the intensity was improved) to 1544 cm^{-1} for the acid-activated films. It is due to the formation of strong ─COOH and ─OH intermolecular bonding that occurred due to the acid activation (─OH excess). This result is also observed with the increase in the spectrum at 1640 cm^{-1} for AF and APF formulation.

5.3.2.4 Thermal properties

The DSC thermograms of the polymers and papain powders are shown in Figure 5.4. An endothermic peak for starch, centered at 47 °C, is associated with the gelatinization of the polymer. Other authors informed peaks around this temperature for potato starch (ALVES et al., 2021; KARLSSON; ELIASSON, 2003; VELÁSQUEZ HERRERA; LUCAS AGUIRRE; QUINTERO CASTAÑO, 2017). The other peak that appeared in the starch analysis is related to water evaporation (at 98 °C). Regarding CMC, an endothermic peak at 108 °C was observed. Some other authors obtained a similar pattern and correlated the peak to loss of water content, but others to the melting temperature of the polymer (AKRAM; TAHA; GHOBASHY, 2016; CHEN et al., 2020; MANDAL; CHAKRABARTY, 2019; SHAHBAZI et al., 2016; TONGDEESOONTORN et al., 2011). For papain powder, their peak of water loss

appeared at 104 °C. But another endothermic peak, at 137 °C, was observed in the result for papain powder. Other studies corresponded the peaks around this region to the start of protein denaturation and melting temperature of papain (LIU et al., 2017; MOREIRA FILHO et al., 2020; VASCONCELOS et al., 2020).

Figure 5.4: Differential scanning calorimetry (DSC) of starch, carboxymethyl cellulose (CMC), papain powders and control film (CF), papain film (PF), acidified film (AF), and acidified papain film (APF).



CF corresponds to film without acid or papain treatment; PF corresponds to film treated with papain solution at pH 8.00; AF corresponds to film treated with 0.10% (w/w) of HCl solution; APF corresponds to film treated with 0.10% (w/w) of HCl solution followed by treatment with papain solution at pH 8.00.

Regarding the films, an endothermic peak at approximately 50 °C and another around 120 °C were observed for all formulations. The first peak is probably correlated to the starch gelatinization, which can be explained by not completing the gelatinization of the starch in the films. The gelatinization process depends on diverse factors, such as temperature, time, and pressure (BAKS et al., 2008; BAUER; KNORR, 2005). The starch solution was maintained at 80 °C for 30 min to produce all CF, PF, AF, and APF films, which probably was insufficient to break down the intermolecular bonds of starch granules completely. The second peak is related to the water loss of the films. The shift observed from the powders to the films is due to the structure that the matrices that difficult the mass water transfer. Moreover, the films with papain immobilization, PF, and APF, showed a band pattern instead of a peak. This difference can be related to the superposition of the water loss and melting temperature of papain in both formulations

5.4 CONCLUSION

In the current research, papain was immobilized by adsorption in starch- and CMC-based films. The pH 8.0 provided the best condition for immobilizing papain in the films (PF), and acid activation with 0.10% (w/v) of HCl provided the best results (APF) for immobilization activity. In addition, the results obtained from Michaelis-Menten plots also showed better results for APF formulation. The acid activation improved the hydrogen bonds for papain adsorption while not affecting the other physic-chemical properties. In general, the characterization showed that papain immobilization resulted in less hydrophilic property of the films than CF and AF formulation. Both PF and APF formulations are thin, flexible, and have good water retention capability and sufficient barrier properties. Based on these results, it is possible to suggest that the obtained films can be used as sheet beauty masks with exfoliation activity.

CHAPTER 6: CONCLUSIONS AND SUGGESTIONS FOR FUTURE STUDIES

6.1 MAIN CONCLUSIONS

This thesis proposed to evaluate the use of papain in cosmetics exfoliants. Despite the use of enzymes in products, during the research for both literature review and experimental data, it was observed that this topic was not explored in depth. The literature review concluded that enzymes can be used as exfoliants, but with some appointments: effects of temperature, pH, enzyme choice, and presence of activators/inhibitors are important when formulating this type of cosmetic and there is a need for more research to demonstrate the feasibility and safety of the use of enzymes in cosmetics.

From the experimental data, it was observed that papain presents proteolytic activity with non- and soluble proteins, such as casein, collagen, and keratin. The concentration of papain demonstrates to be an important point to be investigated when developing a formulation to act on the skin. The presence of cysteine molecules improved the hydrolysis of all studied proteins. Moreover, papain demonstrates to be the ability to act in pH and temperature conditions of human skin at contact times lower than 60 min. Skin sections with topical application of papain presented a similar pattern of exfoliation when acid peel of TCA was applied.

The obtained films made of a blend of starch and CMC presented sufficient thickness and tensile strength and mechanical properties to be used as beauty masks. The films present hydrophilicity properties which are interesting for moisture the skin and be used with other cosmetics, but low solubility in water. When papain was incorporated up until 5% (g of papain per g of polymer) continuous, easy to detach from the molds and handle films were obtained. The incorporation of papain provided more flexible and strong films; the enzyme presented good distribution along the matrices. The films containing papain presented proteolytic activity with casein and skin as substrate and the in vitro exfoliation micrographs of skin showed the exfoliation of this organ. The storage stability showed that the immobilization of papain maintained the activity of the enzyme for at least 90 days, differently from the aqueous (free) form, which lost the activity at nearly 30 days.

Differently, from the direct incorporation of papain in the filmogenic solution, when the adsorption technique was selected to immobilize papain this enabled to increase in the amount of the enzyme in the films of starch and CMC. The use of pH 8.0, near the

isoelectric point of papain, provided the best condition. In addition, the acid activation of the surface of the films with 0.10% (w/v) of HCl improved, even more, the immobilization yield and retained the activity of the films. The possible reason for this was related to the increase of hydroxyl groups. From the Michaelis-Menten plot, it was observed that the acidified film presents a pattern more similar to the native papain form. Concerning characterization, the results showed that both direct adsorptions of papain or the previous acidification did not influence most of the results: the films maintained most of the interesting characteristics for the beauty mask application.

In conclusion, this study solved some gaps in enzymatic exfoliation and demonstrated that papain presents an activity to act in the skin. The immobilization of papain in films showed that the production of active beauty face masks is possible using biopolymers and biotechnology approaches.

6.2 SUGGESTIONS FOR FUTURE STUDIES

The films developed in the present study presented interesting physic-chemical and biological activities to act as beauty face masks. Nevertheless, some investigation can be performed to improve the literature regarding enzymatic exfoliation and the use of enzymes immobilized in cosmetic masks. In this scenario, the following suggestions can be used for future studies:

- Perform different *in vitro* methods for evaluation of exfoliation, principally using histological assays;
- Evaluate the influence of common components of cosmetic and pharmaceutical formulations in the proteolytic activity of papain;
- Study different strategies for immobilizing papain on the surface of the films;
- Evaluation of the biocompatibility of the papain and the starch- and CMC-based films with and without papain immobilization;
- Investigate the *in vivo* application of papain and the beauty face masks in the skin aiming for enzymatic exfoliation.

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