Thermal Characterization and Cytotoxicity of Complexes Formed by Papain and Cyclodextrin

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Abstract Papain is a proteolytic enzyme with restricted applications due to its limited stability. Cyclodextrins are widely used in pharmaceutical formulations once they are able to form complexes with other molecules, improving their stability and bioavailability. The purpose of the present paper was to analyze complexes formed by papain/hydroxypropyl- β -cyclodextrin and papain/ β -cyclodextrin by thermal analysis and cytotoxicity tests to verify their possible interactions and toxicological behavior. Complex solutions were prepared at different molar ratios and collected as a function of time to be lyophilized and analyzed. Results showed changes in endothermic events and cytotoxicity profiles. A complex formation for both complexes is observed; nevertheless, β -cyclodextrin was able to change the enzyme characteristics more efficiently.

Keywords Papain · Cyclodextrin · Cytotoxicity · Differential scanning calorimetry · Fibroblasts · Human keratinocytes

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1 Introduction

Enzymes have been widely used in cosmetics and medicines in recent years, since they are a group of potential active ingredients due to their pharmacological activities [1]. Thus, these biomolecules have been extensively investigated in the search for new therapeutic options with lower toxic effects.

However, most enzymes are not stable, losing biological activity at room temperature in a short period, therefore limiting their applications in pharmaceutical products [1].

Papain is a proteolytic enzyme which belongs to the thiol protease group obtained from *Carica papaya* normally used in wound treatment and for cosmetic reasons. It is composed of a single polypeptide chain of 212 amino acid residues folded into the L and R domain, forming a cavity where its active site is located. The histidine (His-159), aspartic acid (Asp-158) and cysteine (Cys-25) are the residues that compose its active site [2–5].

A natural unfolding mechanism limits the enzyme stability to extemporaneous formulations once it leads to an exposure of the active sites, ceasing its natural activity [6]. A reliable and efficient stabilizing method must be developed allowing the applications of this enzyme in medicines and cosmetic products.

Several substances are able to affect the stability of the folded protein, maintaining the native structure, particularly sugars and polyols [4].

Complexation with cyclodextrins (CD) could improve the stability and bioavailability of the enzyme, since these oligosaccharides are able to form complexes with different molecules, modifying their physical, chemical, and biological characteristics [7], allowing the enzyme to remain active for extended periods of time.

As previously reported by Singh et al. [8], CD derivatives are able to form complexes with free aromatic amino acids at nearly neutral pH, such as tyrosine, phenylalanine, and histidine, depending on their hydrophobicity. These amino acids are naturally present in the enzyme as described by Sumner et al. [9].

Thermodynamic studies allow the assessment of relevant protein properties such as protein stability and function providing good and accurate parameters of analysis for studies concerning isolated proteins [10] as well as protein interactions, such as the present work. Some measurement methods applied nowadays are differential scanning calorimetry (DSC), circular dichroism, tryptophan fluorescence, and changes in tyrosine absorbance [11].

DSC analysis provides fundamental information related to the thermodynamic properties of a protein. It defines the interactions of several molecules with proteins and their effects on the stability [12].

Cosmetics, medications, and related substances must be shown to be as safe as possible for human use, protecting both consumers and workers involved in their production. Alternative nonanimal testing methods are now available, and they demonstrate a strong correlation with *in vivo* assays [13].

Several *in vitro* toxicological methods could be applied to determine the xenobiotic safety using different cell types and cytotoxicity endpoints [14].

These tests could be performed using fibroblasts, the most frequently used cells for validation studies providing reliable results, as well as keratinocytes, the first cells in contact with topical substances, corresponding to 90% of the epidermal layer [13].

The main purpose of this work was to analyze complexes formed by papain and hydroxypropyl- β -CD (HP- β -CD) and papain and β -CD by thermal analysis and cytotoxicity studies in order to verify their possible interaction and toxicological behavior.

Fig. 1 Thermal analysis by DSC of the 1:100 (A) and 1:200 (B) (molar ratio) lyophilized complexes formed by papain/ β -cyclodextrin at 0, 24, and 48 h of complexation

2 Methodology

Papain 30,000 USP purchased from MERCK was diluted in phosphate buffer (pH 6.0) in the presence of L-cysteine hydrochloride monohydrate, acquired from MERCK, to reach a final concentration of 1×10^{-6} M. The CD (HP- β -CD or β -CD) donated by Roquette (France) was added in order to obtain 1:100 and 1:200 molar ratio solutions (papain/CD). The samples were incubated at 50°C under 90 rpm mechanical agitation. Aliquots collected as a function of time (0, 24, and 48 h) were lyophilized using a Micro Modulyo lyophilizer attached to an E2MS BOC EDWARDS vacuum pump in cycles of 30 h and stored at -20°C . A control solution containing only papain was prepared and lyophilized following the same procedures was also tested following the same DSC procedures.

2.1 DSC Analysis

All samples were submitted to DSC analysis, using a DSC2920 TA device with stainless cells (0.7 mL) for solid samples, with a nitrogen flux of 50 mL min^{-1} , from 20°C to 300°C , at a scan rate of $10^{\circ}\text{C min}^{-1}$. For all thermal experiments, 2 mg of the lyophilized complexes were used.

2.2 Cytotoxicity

In order to access the cytotoxicity of the complexes, samples after 48 h of complexation were tested using cultured CCL92 fibroblasts and the *in vitro* MTS (tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium], from Promega) method.

About 10,000 cells were transferred to 96-multiwell plates containing DMEN (Dulbecco's modification of Eagle's medium without L-glutamine), 100 IU penicillin, 4 mM L-glutamine, 10% (*v/v*) newborn calf serum, $100 \mu\text{g mL}^{-1}$ streptomycin, and submitted to incubation with a controlled atmosphere of 37°C with 5% CO_2 and 95% relative humidity for 24 h. Aliquots of 10 μL were used of each sample and control.

The samples were then transferred to the wells in triplicate and the cell viability was observed periodically at 12 and 24 h of contact. After the selected contact time, the medium was removed, and aliquots of 120 μL of the MTS/phenazine methosulfate solution in the described medium were added to each well of the plate, incubated for 2 h, and analyzed at 490 nm, using a Dynatech MR 4000 Elisa reader device.

An experiment using primary 50,000-cultured keratinocytes cells obtained from human skin was also performed following the same procedures, however, using keratinocyte growth medium as a medium. Samples (10 mg) of the lyophilized complexes were used in this test and observed periodically at 24 and 48 h of contact.

The tests were performed under aseptic conditions in a controlled environment using sterilized materials and reagents. The keratinocyte test was submitted to the Ethics Committee, and its protocol number is CEP-UNISO 032/07.

Fig. 2 Thermal analysis by DSC of the 1:100 and 1:200 (molar ratio) lyophilized complexes formed by papain/ β -cyclodextrin and the control group at 0 (a), 24 (b), and 48 h (c) of complexation

3 Results

3.1 Papain/ β -CD Complexes Thermal Profiles Over Time

The DSC analysis showed a profile (Fig. 1a) where the papain/ β -CD complex at a 1:100 molar ratio suffers significant changes from 0 to 24 h. Shifts in all peaks are observed, and a different profile is formed. At 48 h of complexation, an absence of two more peaks held at 51.87°C and 246.43°C is noted as well as shifts in the peaks' temperatures as compared to the complex at 0 h of the complexation.

At 1:200 molar ratio (Fig. 1b), the papain/ β -CD complex presented conformational changes in the endothermic events with the formation of new peaks, at 42.02°C, 214.84°C, and 223.44°C observed at 24 h and a no peak observed at around 159.03°C as compared to the sample at the moment of complexation (0 h). At 48 h, the interaction resulted in the disappearance of the new peaks formed at 24 h, and the profile obtained is similar to the beginning of the experiment; however, the peaks are smaller and presented a shift in the corresponding temperatures.

3.2 Effect of Concentration on Papain/ β -CD Complexes

At the moment of complexation, both papain/ β -CD complex concentrations (Fig. 2a) showed changes in the endothermic event temperatures as compared to the control. The 1:100 complex already presented a different thermal behavior, with new peaks appearing at 51.87°C and 246.43°C.

After 24 h of complexation (Fig. 2b), both concentrations presented an absence of peaks present in the control group at 185.76°C as well as shifts in the peak temperatures. The 1:200 complex also lacks a peak at 147.93°C.

The complexes at 48 h (Fig. 2c) showed a different thermal profile when compared to the control, lacking peaks at 230.91°C and 238.11°C. Significant changes in the conformation of the peak observed at 115°C in the control group were observed for the 1:200 complex.

3.3 Papain/HP- β -CD Complexes Thermal Profiles Over Time

At 1:100 molar ratio, the papain/HP- β -CD complex presented shifts in all endothermic peaks at 24 and 48 h (Fig. 3a). The formation of new peaks at 49.15°C and 249.28°C and the lack of a peak around 188.59°C are noted at 24 h. At 48 h, the complex presented a different thermal behavior, with suppressed peaks at 188.59°C and 216.53°C as compared to the moment of complexation and shifts in the peak temperatures formed after 24 h.

The 1:200 papain/HP- β -CD complex (Fig. 3b) does not suffer significant changes over time. Even though some shifts in the peak temperatures are observed, a similar conformational profile is observed for the samples at 0, 24 and 48 h.

3.4 Effect of Concentration on Papain/HP- β -CD Complexes

At the moment of complexation (Fig. 4a), the 1:100 complex suffered changes in its peaks and an additional peak was observed at 188.59°C. The 1:200 complex presented a similar thermal behavior as the control sample with some shifts in the endothermic event

Fig. 3 Thermal analysis by DSC of the 1:100 (a) and 1:200 (b) (molar ratio) lyophilized complexes formed by papain/hydroxypropyl- β -cyclodextrin at 0, 24, and 48 h of complexation

Fig. 4 Thermal analysis by DSC of the 1:100 and 1:200 (molar ratio) lyophilized complexes formed by papain/hydroxypropyl- β -cyclodextrin and the control group at 0 (a), 24 (b), and 48 h (c) of complexation

temperatures and an additional peak at 252.29°C. After 24 h (Fig. 4b) both complexes presented similar conformational changes if compared to the control sample, with a loss of the peak at 185.76°C. The 1:100 complex also presented an additional peak at 50.03°C.

At 48 h, the samples presented changes in the thermal behavior of the enzyme around 200–250°C (Fig. 4c) and suppressed peaks at 223.83°C and 230.91°C for the 1:100 sample and 230.91°C for the 1:200 complex, respectively. The 1:100 sample still presents an additional peak formed at 24 h (50.03°C) that at 48 h was observed at 33.65°C. The 1:200 sample presented more significant changes as compared to the 1:100 complex, with respect to the control group, presenting more shifts in the peak temperatures.

3.5 Cytotoxicity

The test performed with fibroblasts indicated that the complexed enzyme with both CDs showed less cytotoxicity than the isolated papain at 12 and 24 h of contact (Fig. 5). All results were above 50% cell viability considering the control group.

At 24 and 48 h (Fig. 6), the complexes presented a different cytotoxic behavior as compared to the controls, presenting an increased toxicity in the keratinocyte assay.

4 Discussion and Conclusions

DSC plays a remarkable role in studies involving biomolecule stabilization [12]. According to DSC analysis, change of the characteristic temperatures of the endothermic events were noted for both complexes, and a disappearance of some endothermic peaks was observed.

The complexity of the thermal profiles obtained for the complexes was expected since papain domains (L and R domain) vary in chain size and amino acid quantity, thus generating different endothermic peaks that correspond to the unfolding of each domain, once they are known to unfold at different temperatures, not independently, but successively. In addition, the presence of an intermediate state formed during the protein denaturation process contributes to this fact [4, 15].

For both complexes, the 1:200-molar ratio samples presented more relevant changes when compared to the control, indicating a possibly more effective complex formation than the 1:100 complexes.

This behavior is expected since papain's molecular size is larger than that of CD requiring several CD molecules in order to stabilize the enzyme. An inclusion complex formation with the entire molecule is sterically impossible, but an interaction among the several CD molecule cavities with specific areas of the protein, such as hydrophobic amino acid residues, can be achieved [16].

Particularly, the first domain (residues 1–100) has a polypeptide chain made up of five hydrophobic side chains, and the second (111–212) possesses a larger hydrophobic core formed by 12 side chains indicating that both domains are involved in the complexation process [15], providing an explanation for the effect of CD concentration on the different thermal behaviors observed for the complexes at 1:100 and 1:200 molar ratio.

Fig. 5 A comparison between fibroblast cell viability of isolated papain/ β -cyclodextrin, hydroxypropyl- β cyclodextrin, and the complexes formed after 48 h of complexation by papain/ β -cyclodextrin and papain/hydroxypropyl- β -cyclodextrin at 1:100 and 1:200 molar ratio after 12 and 24 h of contact

Both CDs were able to interact with the enzyme, causing significant thermal changes. The free energy required to accommodate the unfolded protein molecule in the solvent cavities is higher than the necessary energy for the folded state; therefore, an increase in stability and predominance of the folded state is favored in the presence of sugars. This is related to the effect of water surface tension on preferential hydration of sugars, where

Fig. 6 A comparison between the keratinocyte viability of isolated papain, β -cyclodextrin, hydroxypropyl- β cyclodextrin, and the complexes formed after 48 h of complexation by papain/ β -cyclodextrin and papain/hydroxypropyl- β cyclodextrin at 1:100 and 1:200 molar ratio after 24 and 48 h of contact

the cavity formation for unfolded molecules will be larger, thus presenting lower stability [17, 18].

However, the profile observed for the β -CD complex showed a more stable complex formation indicated, by a higher absence of peaks. As reported by Del Valle [19], β -CD is more hydrophobic than HP- β -CD, and thus has more affinity for the hydrophobic amino acid residues present in the enzyme. Even though some residues present in the active site are hydrophobic and possibly involved in the complexation, it is relevant to consider the fact that this process is a dynamic equilibrium instead of a permanent binding, since no covalent bonds are broken or formed. The release of enthalpy-rich water molecules from the CD's cavity is the driving force behind the complex formation [19–21].

Considering that, at 0 and 24 h of incubation, the complexes showed changes in the profiles and even loss of peaks, only at 48 h were consistent conformational changes observed, indicating a more stable complex.

Even though fibroblasts are not the first cells to be in contact with topic agents, they are extensively used in cytotoxicity studies. Among several methods applied, the MTS was the selected one, since it requires fewer steps during analysis. The method is based on the ability of viable cells to convert a soluble tetrazolium salt to a formazan product that could be measured by spectroscopic assay [22]. These tests do not replace *in vivo* assays but are able to estimate the toxic dose of a certain drug or medicine.

The experiments performed at 12 and 24 h showed a very similar profile where all samples were above 50% cell viability, indicating that the complex is not cytotoxic.

As compared to the isolated enzyme, the complexes presented enhanced behavior with less toxic effects, confirming that the interaction resulted in changes in the enzyme characteristics at the tested concentrations and conditions. These concentrations were lower than in the samples used for the keratinocyte test since these cells are more sensitive.

According to Moraes et al. [23], HP- β -CD does not show any cytotoxic effect over fibroblast cells as confirmed through our results, showing no interference.

Thus, the cytotoxicity presented for the complexes at 1:200 is possibly due to residual papain activity.

At higher concentrations, papain behaved as expected due to its proteolytic characteristics and cytotoxic behavior at 24 h. However, at 48 h, the enzyme caused an increase in cell viability as previously described by Lopes [24]. This profile is not observed for the complexed enzyme where the 1:100-molar ratio complexes were less toxic than the 1:200.

It is relevant to note that the concentrations of the samples used for the keratinocytes were much higher and lyophilized, which prevents enzyme degradation, which may explain the different profiles obtained for the different cells. This strategy was employed as a screening of the possible dosage limit.

The next step involves the determination of IC₅₀, defining the concentration responsible for 50% of cell death, and assays to determine their activity with specific substrate stability since the complex formation does not guarantee that the enzyme is stabilized, and there is the possibility that not all the amino acid residues are complexed, and thus not blocking the natural unfolding mechanism leading to inactivation.

5 Conclusion

In summary, we find that, for both the DSC results and cytotoxicity studies, the 1:200-molar ratio complexes for both CDs presented a more stable complex formation; however, β -CD

presented advantages over HP- β -CD. Activity assays still need to be performed in order to evaluate the complexation process efficiency.

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References

1. Sim, Y.C., Lee, S.G., Lee, D.C., Kang, B.Y., Park, K.M., Lee, J.Y., Kim, M.S., Chang, I.S., Rhee, J.S.: Stabilization of papain and lysozyme for application to cosmetic products. *Biotechnol. Lett.* **22**, 137–140 (2000). doi:[10.1023/A:1005670323912](https://doi.org/10.1023/A:1005670323912)
2. Kamphuis, I.G., Kalk, K.H., Swarte, M.B.A., Drent, H.J.: Structure of papain refined at 1.65 angstrom resolution. *J. Mol. Biol.* **179**, 233–256 (1984). doi:[10.1016/0022-2836\(84\)90467-4](https://doi.org/10.1016/0022-2836(84)90467-4)
3. O'Neil, M.J.: The Merck Index, 13th edn. Merck Research Laboratories, Whitehouse Station, NJ (2001)
4. Sathish, H.A., Kumar, P.R., Prakash, V.: Mechanism of solvent induced thermal stabilization of papain. *Int. J. Biol. Macromol.* **41**, 383–390 (2007). doi:[10.1016/j.ijbiomac.2007.05.009](https://doi.org/10.1016/j.ijbiomac.2007.05.009)
5. Zhuang, P., Butterfield, D.A.: Denaturation studies of active-site labeled papain using electron paramagnetic resonance and fluorescence spectroscopy. *Biophys. J.* **60**, 623–628 (1991)
6. Rialdi, G., Battistel, E.: Unfolding mechanism and stability of immobilized papain. *J. Therm. Anal.* **47**, 17–25 (1996). doi:[10.1007/BF01982682](https://doi.org/10.1007/BF01982682)
7. Legendre, J.Y., Rault, I., Petit, A., Luijten, W., Demuynck, I., Horvath, S., Ginot, Y.M., Cuine, A.: Effects of β -cyclodextrins on the skin: implications for the transdermal delivery of pibredil and a novel cognition enhancing-drug, S-9977. *Eur. J. Pharm. Sci.* **3**, 311–322 (1995). doi:[10.1016/0928-0987\(95\)00020-0](https://doi.org/10.1016/0928-0987(95)00020-0)
8. Singh, M., Sharma, R., Banerjee, U.C.: Biotechnological applications of cyclodextrins. *Biotechnol. Adv.* **20**, 341–359 (2002). doi:[10.1016/S0734-9750\(02\)00020-4](https://doi.org/10.1016/S0734-9750(02)00020-4)
9. Sumner, I.G., Harris, G.W., Taylor, M.A.J., Pickersgill, R.W., Owen, A.J., Goodenough, W.: Factors effecting the thermostability of cysteine proteinases from *Carica papaya*. *Eur. J. Biochem.* **214**, 129–134 (1993). doi:[10.1111/j.1432-1033.1993.tb17904.x](https://doi.org/10.1111/j.1432-1033.1993.tb17904.x)
10. Rialdi, G., Battistel, E.: Thermodynamics of proteins in unusual environments. *Biophys. Chem.* **126**, 65–79 (2006). doi:[10.1016/j.bpc.2006.05.029](https://doi.org/10.1016/j.bpc.2006.05.029)
11. Pollizi, K.M., Bommarius, A.S., Broering, J.M., Chaparro-Riggers, J.F.: Stability of biocatalysts. *Curr. Opin. Chem. Biol.* **11**, 220–225 (2007). doi:[10.1016/j.cbpa.2007.01.685](https://doi.org/10.1016/j.cbpa.2007.01.685)
12. Shnyrov, V.L., Sanchez-Ruiz, J.M., Boiko, B.N., Zhadan, G.G., Permyakov, E.A.: Applications of scanning microcalorimetry in biophysics and biochemistry. *Thermochim. Acta* **302**, 165–180 (1997). doi:[10.1016/S0040-6031\(97\)00238-4](https://doi.org/10.1016/S0040-6031(97)00238-4)
13. National Institutes of Health: Guidance document on using *in vitro* data to estimate *in vivo* starting doses for acute toxicity. No. 01-4500. <http://www.epa.gov/chemrtk/pubs/general/nih2001b.pdf>, August (2001)
14. Freshney, R.I.: *Culture of Animal Cells: A Manual of Basic Technique*, 5th edn. Wiley-Liss, New York (2005)
15. Hernández-Arana, A., Soriano-García, M.: Detection and characterization by circular dichroism of a stable intermediate state formed in the thermal unfolding of papain. *Biochim. Biophys. Acta* **954**, 170–175 (1988)
16. Fernández, M., Frago, A., Cao, R., Villalonga, R.: Stabilization of α -chymotrypsin by chemical modification with monoamine cyclodextrin. *Process Biochem.* **40**, 2091–2094 (2005). doi:[10.1016/j.procbio.2004.07.023](https://doi.org/10.1016/j.procbio.2004.07.023)
17. Lee, J.C., Timasheff, S.N.: The stabilization of proteins by sucrose. *J. Biol. Chem.* **256**, 7193–7201 (1981)
18. Arakawa, T., Timasheff, S.N.: Stabilization of protein structure by sugars. *Biochemistry* **21**, 6536–6544 (1982). doi:[10.1021/bi00268a033](https://doi.org/10.1021/bi00268a033)
19. Martín Del Valle, E.M.: Cyclodextrins and their uses: a review. *Process Biochem.* **39**, 1033–1046 (2004). doi:[10.1016/S0032-9592\(03\)00258-9](https://doi.org/10.1016/S0032-9592(03)00258-9)
20. Schneiderman, E., Stalcup, A.M.: Cyclodextrins: a versatile tool in separation science. *J. Chromatogr. B* **745**, 83–102 (2000). doi:[10.1016/S0378-4347\(00\)00057-8](https://doi.org/10.1016/S0378-4347(00)00057-8)
21. Szejtli, J.: Introduction and general overview of cyclodextrin chemistry. *Chem. Rev.* **98**, 1743–1753 (1998). doi:[10.1021/cr970022c](https://doi.org/10.1021/cr970022c)

22. Promega: Technical Bulletin no. 169: Cell titer 96[®] aqueous non-radioactive cell proliferation assay. Updated 12/2006. http://www.promega.com/enotes/applications/sap0017_print.htm. Cited 5 Dec 2005 (2005)
23. Moraes, C.M., Araujo, D.R., Issa, M.G., Ferraz, H.G., Yokaichiya, F., Franco, M.K.K.D., Mazzaro, I., Lopes, P.S., Gonçalves, M.M., de Paula, E., Fraceto, L.F.: Inclusion complex of S(-) bupivacaine and 2-hydroxypropyl- β -cyclodextrin: study of morphology and cytotoxicity. *Rev. Ciênc. Farm. Básica Apl.* **27**(3), 207–212 (2006)
24. Lopes, P.S.: Avaliação da eficácia e segurança da papaína como promotor de absorção cutâneo utilizando técnicas biofísicas e cultura celular de queratinócitos humanos. Ph.D. thesis, University of São Paulo (2003)