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ABSTRACT

IMMUNEPOTENT CRP is a dialyzable leukocyte extract obtained from bovine spleen with immunomodulatory and antitumour properties; therefore, when administrated as an adjuvant therapy for cancer patients, it has increased their survival and quality of life. The bioavailability of any orally administered compound can be reduced due to gastrointestinal enzymes. In this study, we evaluated if IMMUNEPOTENT CRP is resistant to the treatment with different enzymes (proteases, nucleases, polysaccharide-degrading enzymes or lipase), using as parameters for biological activity measurement its *in vitro* antitumour and antioxidant properties and *in vivo* the antitumour effect of IMMUNEPOTENT CRP treated with proteinase K. In conclusion, we consider necessary to include the antioxidant and cytotoxic activity on the MCF-7 cancer cell line as parameters for the quantitative determination of biological activity or potency tests for batch release. Additionally, the results showed that different enzymatic treatments do not affect the antitumour and antioxidant activities of IMMUNEPOTENT CRP *in vitro*, suggesting that this product can be administered orally without any loss of biological activity. Furthermore, IMMUNEPOTENT CRP treatment with proteinase K increases the antitumour activity *in vivo*.

Introduction

In 1955, doctor Sherwood Lawrence described for the first time that delayed type IV hypersensitivity can be transferred from an immunocompetent individual to a naive recipient by administration of a dialyzable leukocyte extract (DLE). Lawrence termed the unknown molecule or group of molecules responsible for this effect 'transfer factor' [1]. The dialyzable extract (DLE) is a heterogeneous mixture of substances resulting from the disruption and dialysis (<12 kDa) of leukocytes into an aqueous solution. It is derived from different sub-populations of leukocytes, each one with different functions and molecules; therefore the DLE has many biological effects on the immune system, not all of them immunity transference related [2]. Due to its immunomodulatory properties, the DLE containing transfer factor has been used for the treatment of many immune system related pathologies and cancer [3]. Immunotherapy has become one of the standard treatments for cancer; therefore immunomodulatory and adjuvant substances are being investigated for this purpose, biological compounds

included. However, it is important to optimize their formulation to guarantee efficacy, safety, and stability in any form of administration [4,5]. IMMUNEPOTENT CRP (ICRP) is a biological compound obtained from the disruption of bovine spleen leukocytes containing transfer factor with versatile activities. It has been proved to modulate cytokine production [6], increase the number of immunocompetent cells (CD4+, CD8+, CD56+) [7], protect the bone marrow from aggressive side effects of chemotherapy [8] and induce differentiation of leukemia blast cells [9]. *In vitro* studies also show that ICRP is toxic to cancer cell lines [10] and has strong antioxidant properties [6]. Overall its administration as an adjuvant therapy to patients with cancer improves their quality of life and survival [7,11]. In addition to its efficacy, ICRP is reported free of adverse effects in humans. It is a non-pyrogenic substance, with no HLA antigens and it can be lyophilized and stored for a long time without losing its biological properties [10]. However, there is a lack of information regarding the potential degradation of ICRP or any other DLE once it is administered into the body [12]. The most desirable route of administration for any

drug therapy is oral, particularly for the treatment of chronic conditions. But many compounds are susceptible to the conditions of the gastrointestinal tract. Therefore, any biological compound should be exposed to conditions that may compromise the quality and stability, for example, low pH and hydrolytic enzymes, and tested for any activity decrease [13]. In the present study, we focused on the resistance of the ICRP to enzymatic treatment over a specific type of biomolecule (proteins, lipids, carbohydrates or nucleic acids) using the antioxidant and antitumour *in vitro* properties of ICRP as potency tests of biological activity. Furthermore, we tested the antitumour properties of ICRP and ICRP treated with proteinase K *in vivo*. The information obtained will contribute to improve the ICRP formulation and oral administration without loss of biological activity.

Materials and methods

IMMUNEPOTENT CRP

ICRP was produced by the Laboratory of Immunology and Virology, Faculty of Biological Sciences, University Autonomous of Nuevo León (UANL) (San Nicolás de los Garza, Mexico). ICRP is dialyzed, lyophilized and pyrogen-free. One unit of ICRP has been defined as the product obtained from 15×10^8 bovine spleen cells.

Enzymes

The following enzymes were used in this study. Proteases: Collagenase (Sigma, Chemical Company, St. Louis MO, USA), pepsin (0.8% pepsin, 0.2 N HCl) (DAKO, São Paulo, Brasil), neutral protease (Worthington Biochemical Corporation, Lakewood, New Jersey, USA), proteinase K (MP Biomedical, Santa Ana, California, USA), trypsin (Sigma, Chemical Company, St. Louis MO, USA) and validase (Valley Research). Nucleases: deoxyribonuclease I (Sigma, Chemical Company, St. Louis MO, USA) and ribonuclease A (US Biologicals Salem, Massachusetts, USA). Polysaccharide degrading enzymes: α-amylase (Sigma, Chemical Company, St. Louis MO, USA) and lysozyme (MP Biomedical, Santa Ana, California, USA). Lipase: lipozyme (Novo Industrials, Copenhagen, Denmark).

Cell lines

Breast carcinoma MCF7 (ATCC® HTB-22™) cell line was obtained from ATCC (American Type Culture Collection, Manassas, VA, USA) and cultured in DMEM medium with 10% bovine serum and 1% of antimycotic-antibiotic (penicillin, streptomycin and amphotericin B) (Sigma,

Chemical Company, St. Louis MO, USA), and incubated in atmosphere with 5% CO_2 at 37 °C. Murine lymphoma cell line L5178-R (LY-R) (ATCC® CRL-1722™) was obtained from ATCC (American Type Culture Collection, Manassas, VA. USA) and maintained as transplantable ascites tumour in BALB/c mice. The pattern of tumour growth was consistent and reproducible.

Animals

Female (6–8 weeks old) BALB/c mice housed in separate stainless steel cages (5 mice per cage) at constant temperature (25 °C) with light and dark cycles of 12 h, access to water and feed *ad libitum* with rodent balanced diet (Harlan Teklad). All procedures of this study were approved by the Animal Bioethics Committee of the Laboratory of Immunology and Virology of the Biological Sciences Faculty of the National University of Nuevo León (CEIBA).

Determination of proteases enzymatic activity

To confirm the proteases enzymatic activity, an ovalbumin solution (Sigma, Chemical Company, St. Louis MO, USA) was prepared and allotted as follows: ovalbumin, ovalbumin with collagenase, ovalbumin with pepsin, ovalbumin with neutral protease, ovalbumin with proteinase K, ovalbumin with trypsin and ovalbumin with validase. The aliquots were incubated at 37 °C for 2 h and protein concentration was determined by the DC™ Protein Assay (BIO-Rad, Hercules, California, U.S.A.).

Determination of nucleases enzymatic activity

To confirm the nucleases enzymatic activity, we extracted plasmid DNA from *E. coli* DH5-Alpha following the Ausbel minipreparation protocol with a minor modification: we did not add the RNase into the preparation to obtain bacterial RNA as well. The resultant nucleic acid preparation was quantified by spectrophotometry using the NanoDrop™ 2000 (Life Technologies; Thermo Fisher Scientific, Inc., Waltham, MA, USA) and allotted as follows: nucleic acid preparation, nucleic acid preparation with DNAse, nucleic acid preparation with RNase and nucleic acid preparation with DNAse and RNase. All aliquots were incubated at room temperature (25 °C) for 10 min, followed by electrophoresis in agarose gel (0.8%) and ethidium bromide staining (10 μg/mL). Universal Hood II Electrophoresis Imaging Cabinet (BIO-Rad, Hercules, California, U.S.A.) was used for gel visualization and imaging.

Determination of polysaccharide-degrading enzymes activity

To confirm the α-amylase activity, we prepared agar (0.15 g) (BD Biosciences) with starch (0.1 g) in 10 mL of ultrapure water and poured the preparation in 2 plates of 3 cm diameter (2 mL per plate). Once the agar solidified, we added α-amylase (1 mg/mL) to one of the plates. Both plates were incubated at 37 °C for 1 h and stained with Lugol (100 μL). To confirm the activity of lysozyme, we prepared agar (0.15 g) (BD Biosciences) with lyophilized *Micrococcus lysodeikticus* (0.1 g) in 10 mL of ultrapure water and poured the preparation in 2 plates of 3 cm diameter (2 mL per plate). Once the agar solidified, we added lysozyme to one of the plates, and both plates were incubated at 37 °C for 1 h. Clear zones in the agar indicated lysozyme activity.

Determination of lipase activity

To confirm the lipozyme activity, we prepared agar (0.15 g) (BD Biosciences) with oil (2 mL) in 10 mL of ultrapure water, and poured the preparation in 2 plates of 3 cm diameter (2 mL per plate). Once the agar solidified, we added lipozyme to one of the plates. Both plates were incubated at 37 °C for 1 h and stained with Sudan III (Sigma, Chemical Company, St. Louis MO, USA).

IMMUNEPOTENT CRP treatment with enzymes

Several aliquots of ICRP (5 units) were diluted in DMEM medium (1 mL). Each aliquot was then treated for 24 h with a specific enzyme at the pH, concentration and temperature established by the manufacturer as described in Table 1. Finally, all aliquots were heated in a water bath at 100 °C for 10 min to inactivate the enzymes, pH was adjusted to 7, and ICRP was diluted to a final concentration of 2 units/mL in DMEM medium supplemented with fetal bovine serum (10%).

Table 1. Protocol of enzymatic treatment of IMMUNEPOTENT CRP.

| Enzyme	Concentration	pH	Temperature (°C)	Time (h)
Collagenase	1 mg/mL	7.4	37	24
Pepsin	100 μL/mL	2.0	37	24
Neutral protease	5 mg/mL	7.5	37	24
Proteinase K	10 mg/mL	7.5	37	24
Trypsin	1 mg/mL	7.0	37	24
Validase	5 mg/mL	3.0	37	24
DNase I	70 units/mL	7.0	25	24
RNase A	3.75 μL/mL	7.6	37	24
α-amylase	1 mg/mL	6.9	40	24
Lysozyme	10 mg/mL	6.2	37	24
Lipozyme	4.3 mg/mL	8.0	55	24

Cell viability assay

The dose–response curve of ICRP-treated MCF7 cancer cell line was obtained by the resazurin assay (Resazurin sodium salt; Sigma Chemical Company, St. Louis MO, USA). MCF7 cells were seeded in a 96-well plate at a density of 5000 cells per well and incubated at 37 °C in 5% CO_2 atmosphere for 24 h. The cells were exposed to different concentrations of IMMUNEPOTENT CRP treated or not treated with enzymes (0.2–2.0 units/mL) and incubated at 37 °C in 5% CO_2 atmosphere for 24 h. The medium was removed and 100 μL of DMEM supplemented with 10% bovine serum and 20 μL of resazurin were added. The plate was incubated at 37 °C in 5% CO_2 atmosphere for 12 h. The fluorescence was measured at 530 nm excitation wavelength and 590 nm emission wavelength with the Synergy HT^{TM} (BioTek Instruments, Inc., Winooski, VT, USA) plate reader.

MTT antioxidant assay

Aliquots of 200 μL/well of IMMUNEPOTENT CRP treated or not treated with enzymes were pipetted to a 96-well plate and 100 μL of 3-(4,5-dimethylthiazol-2-yl)−2,5-diphenyltetrazolium bromide [MTT] (1 mg/mL) were added. The reaction mixture was then incubated at 37 °C for 2 h, and the absorbance was measured at 570 nm wavelength with the Synergy HT^{TM} (BioTek Instruments, Inc., Winooski, VT, USA) plate reader. Each sample was assayed in triplicate.

Modified mouse air pouch model for evaluation of the antitumour activity of IMMUNEPOTENT CRP

Air pouches were formed as described by Gaspar et al. [14], with modifications. Briefly, 5 mL of sterile air were injected subcutaneously into the shaved back of the mice; after 3 days, another 3 mL of sterile air were injected once again into the back of the animals. Three days later, L5178Y-R viable cells (2×10^6 in 0.5 mL of PBS) were injected subcutaneously into the air pouch of every mouse and these were randomly divided into 5 groups ($n = 3$, each group) for therapy administration: PBS (group 1), 5-fluorouracil (20 mg/kg) (group 2), ICRP – 3 units (group 3), ICRP – 5 units (group 4) and ICRP treated with proteinase K (group 5). Therapy began on the same day of L-5178Y-R tumour cells inoculation; all treatments were administered daily for 9 days into the air pouch. The day after the last injection, the mice were anesthetized with an intraperitoneal injection of a solution containing 100–200 mg/kg mouse body weight of ketamine and 5–16 mg/kg mouse body weight of
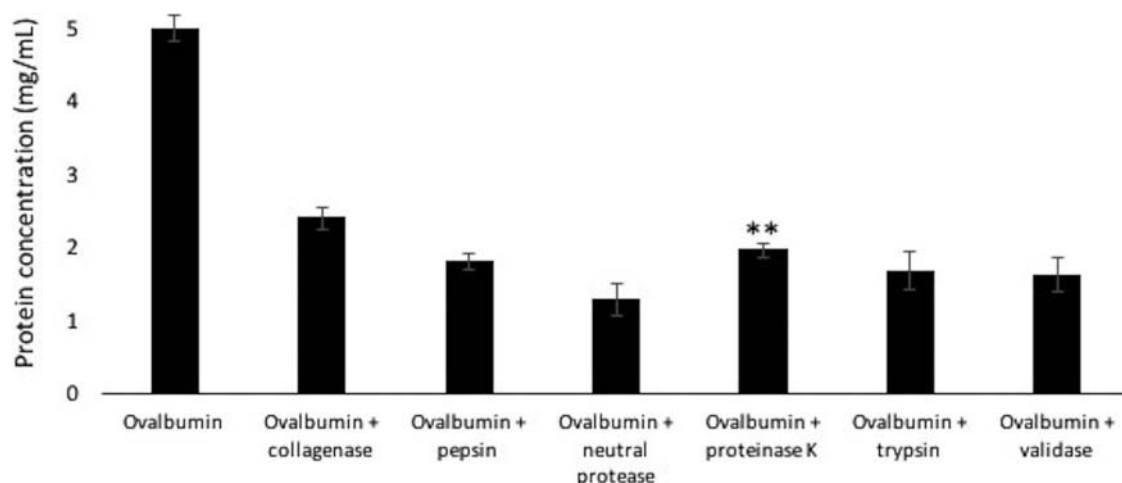
Figure 1. Protease activity evaluation.
Note: Ovalbumin solution (5 mg/mL) was incubated with different proteases at 37 °C for 2 h. Values are means from three independent experiments. **$P < 0.01$, significant difference (*post hoc* Dunnet test).

xylazine, and were sacrificed by cervical dislocation, as approved by the Animal Research and Welfare Ethics Committee of the Faculty of Biological Sciences (CEIBA) of UANL. For therapy evaluation (observable tumour progression) the tumour foci and tumour weight were taken into account.

Statistical data analysis

All experiments were performed in triplicate and are presented as average and standard deviation ($\pm$SD). Statistical analysis was performed using SPSS 22.0 statistical software (SPSS, Inc., Chicago, IL, USA) and included analysis of variance followed by *post hoc* Dunnett's or Tukey tests. Values of $p < 0.05$ were considered to indicate a statistically significant differences.

Results and discussion

IMMUNEPOTENT CRP has improved the overall condition and life expectancy of cancer patients when administered as an adjuvant therapy [11]. As with any other biological compound, the oral route is the most desirable administration pathway [5]; however there are no reports of the preservation or degradation of the biological activities of IMMUNEPOTENT CRP once it is exposed to the harsh conditions of the gastrointestinal tract, such as low pH, changes in temperature and especially hydrolytic activity of enzymes. Other dialyzable leukocyte extracts have been exposed to enzymatic treatments in the past, and the reported results vary [15,16]. By means of these studies, the authors suggested that the immunity transference is RNA related and that the *in vitro* rosette formation is due to the protein fraction from the extract [15,16]. The enzymes used in these studies were

nucleases and proteases only, and no other biological activities were evaluated. In the present study, we exposed IMMUNEPOTENT CRP to the hydrolytic activity of proteases (collagenase, pepsin, neutral protease, proteinase K, trypsin and validase), nucleases (DNAse I and RNase A), polysaccharide-degrading enzymes (α-amylase and lysozyme) and a lipase (lipozyme).

To confirm that our enzymes were active, we tested them over their known substrates. For evaluation of proteases functionality, we used ovalbumin (5 mg/mL) as our protein substrate, finding a highly significant (**$p < 0.01$) decrease in the ovalbumin concentration dependent on the enzymatic treatment: collagenase (2.39 mg/mL), pepsin (1.80 mg/mL), proteinase K (1.95 mg/mL), neutral protease (1.28 mg/mL), trypsin (1.67 mg/mL) and validase (1.62 mg/mL) compared with control (untreated ovalbumin) (5 mg/mL) (Figure 1). For nucleases (DNase I and RNase A) evaluation, we employed plasmid DNA and bacterial RNA as nucleic acid substrates, observing degradation of both substrates by their respective enzymes (Figure 2). To determine the functionality of α-amylase, we evaluated the degradation of starch by lugol staining (Figure 3), and for lysozyme, the degradation of lyophilized *M. lysodeikticus* by change of turbidity (Figure 4). We observed substrate degradation in both cases. To test the lipozyme functionality, we used agar with oil emulsions and corroborated the substrate degradation by Sudan III staining (Figure 5). According to our results, all enzymes used in the present study were functional.

MCF7 cells were exposed to heat-inactivated enzymes using the same concentrations as the ones used with IMMUNEPOTENT CRP. There was no significant difference ($P < 0.05$) between the viability of the treated and non-treated MCF7 cells (Table 2), corroborating that
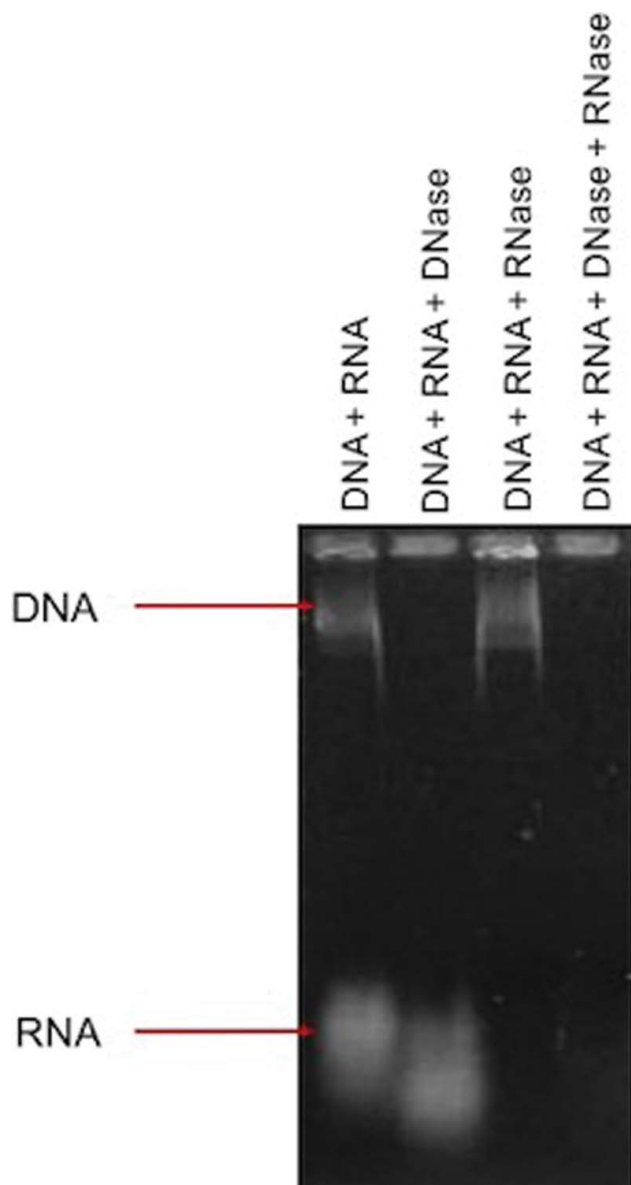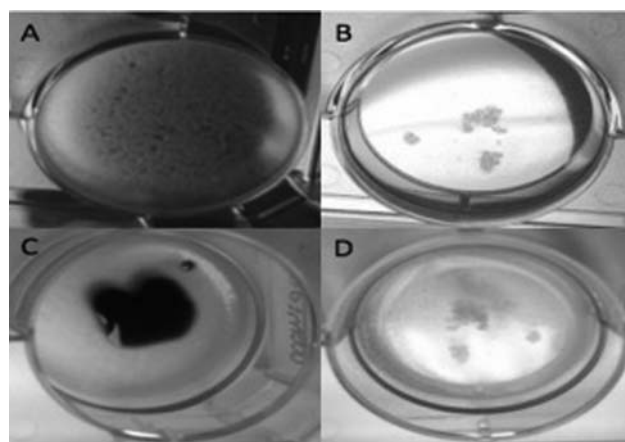
Figure 2. Nuclease activity evaluation.
Note: Agarose gel (0.8%) electrophoresis and bromide ethidium staining (10 μg/mL) of plasmid DNA and bacterial RNA treated with DNase I and RNase A, respectively.
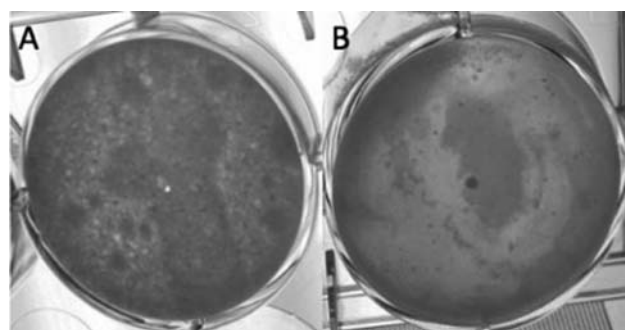

Figure 3. α-Amylase activity evaluation.
Note: Agar containing starch (A) and agar containing starch treated with α-amylase (B) were prepared and incubated for 1 h at 37 °C and stained with Lugol (100 μL) evidencing starch presence in the agar not treated with α-amylase (C) and starch degradation in the agar treated with the enzyme (D).


Figure 4. Lysozyme evaluation activity.
Note: Agar containing lyophilized *M. lysodeikticus* (A) and agar containing lyophilized *M. lysodeikticus* and treated with lysozyme (B) were prepared and incubated for 2 h at 37 °C. *M. lysodeikticus* degradation was evidenced by change in the turbidity of the agar.

heat-inactivated enzymes do not affect MCF7 cells viability.

IMMUNEPOTENT CRP treated with enzyme or not treated decreased the viability of MCF7 cells in a dose-dependent manner ($p < 0.05$) (Table 3). However, there were significant differences ($p < 0.05$) in the lethal doses obtained at the concentrations of LD$_{25}$ (1 U/mL) and LD$_{50}$ (1.2 U/mL) between treatments. IMMUNEPOTENT CRP treated with proteinase K showed an increased antitumour activity when compared to non-treated IMMUNEPOTENT CRP (*$p < 0.05$). According to Segura-Campos et al. [17], enzymatic digestion can liberate fragments with higher biological activity than the precursor

compound. There were no significant differences (*$p < 0.05$) in the cellular viability decrease at the absolute lethal dose, LD$_{100}$ (1.8 U/mL), between treatments (Table 3).

Regarding the antioxidant activity, there was a significant difference (*$p < 0.05$) between the DMEM medium and the IMMUNEPOTENT CRP treated or not with enzymes. However, there was no significant difference (*$P < 0.05$) in the antioxidant activity values between IMMUNEPOTENT CRP treated and not treated with enzyme (Figure 6).

Our results showed that the *in vitro* biological activities (antitumour and antioxidant) of IMMUNEPOTENT CRP are resistant to the enzymatic treatments used. This study is important because the bioavailabiltiy of compounds administered by the oral route can be limited due to pre-systemic metabolism by hydrolytic activity of enzymes present the gastrointestinal tract, resulting in a
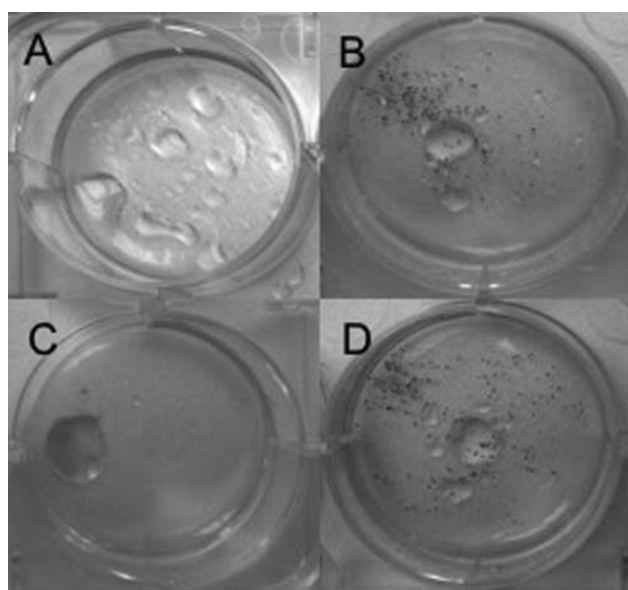
Figure 5. Lipozyme activity evaluation.
Note: Agar containing oil emulsions (A) and agar containing oil emulsions and treated with lipozyme (B) were prepared and incubated for 1 h at 37 °C and stained with Sudan III evidencing oil presence in the agar not treated with lipozyme (C) and oil degradation in the agar treated with lipozyme (D).

reduced biological effect [18], such as reported by Kupferschmidt et al. [19] and Tuteja et al. [20]. The bioavailability of saquinavir and tacrolimus, respectively, was less than 20% when administered by the oral route [19,20]. Moreover, as part of our commercial production strategy, we consider it necessary to include the antioxidant and cytotoxic activity on the MCF-7 cancer cell line as parameters for the quantitative determination of biological activity or potency tests for the batch release.

Table 2. Effect of inactivated enzymes on the viability of MCF7 cells.

Treatment	Viability (%)		
	LD_{25} (1 U/mL)	LD_{50} (1.2 U/mL)	LD_{100} (1.8 U/mL)
Control	100 ± 1.72	100 ± 3.49	98.28 ± 0.07
Collagenase	99.63 ± 5.02	98.67 ± 3.1	98.32 ± 0.38
Neutral protease	98.03 ± 1.15	100 ± 2.14	100 ± 3.62
Pepsin	97.65 ± 1.61	99.74 ± 2.53	99.14 ± 0.64
Proteinase K	100 ± 4.20	97.35 ± 3.10	97.56 ± 2.61
Trypsin	100 ± 2.92	98.87 ± 1.74	100 ± 1.54
Validase	100 ± 2.38	98.5 ± 1.86	97.63 ± 1.33
DNAse I	95.18 ± 2.00	99.76 ± 1.81	98.85 ± 2.15
RNAse A	99.25 ± 0.88	94.31 ± 4.04	100 ± 2.42
α-amylase	100 ± 3.92	99.89 ± 1.82	100 ± 1.33
Lysozyme	99.22 ± 1.09	97.40 ± 0.9	99.88 ± 2.25
Lipozyme	97.99 ± 1.44	97.85 ± 3.42	98.90 ± 3.19

Table 3. *In vitro* antitumour effect of IMMUNEPOTENT CRP treated and not treated with enzyme on MCF7 cell line viability.

| Treatment	Viability (%)		
	LD_{25} (1 U/mL)	LD_{50} (1.2 U/mL)	LD_{100} (1.8 U/mL)
ICRP	73 ± 1.72	43.47 ± 3.49	0.14 ± 0.07
ICRP + collagenase	62.29 ± 5.02*	52.14 ± 7.1	1.07 ± 0.38
ICRP + neutral protease	55.23 ± 4.33*	32.43 ± 4.07*	1 ± 0.09
ICRP + pepsin	66.38 ± 1.72*	38 ± 3.49*	0.58 ± 0.06
ICRP + proteinase K	19.38 ± 4.92*ᵃ	11.10 ± 1.53*ᵃ	1.07 ± 0.66
ICRP + trypsin	64.4 ± 1.72*	23.13 ± 3.49*	1.3 ± 0.06
ICRP + validase	78.51 ± 3.36	64.49 ± 4.41*	0.77 ± 0.179
ICRP + DNase I	30.15 ± 0.84*	27.14 ± 1.06*	1.01 ± 0.37
ICRP + RNase A	35.38 ± 2.08*	25.32 ± 0.73*	1.07 ± 0.38
ICRP + α-amylase	75.49 ± 3.52	31.78 ± 3.55*	1.23 ± 1.17
ICRP + lysozyme	78.38 ± 4.65	52.59 ± 3.94	0.41 ± 0.27
ICRP + lipozyme	39.53 ± 4.43*	22.38 ± 2.67*	0.73 ± 0.42

* $P < 0.05$.

We also tested the *in vivo* antitumour activity of IMMUNEPOTENT CRP and IMMUNEPOTENT CRP treated with proteinase K, finding that the three units dose did not affect the tumour foci and tumour weight when
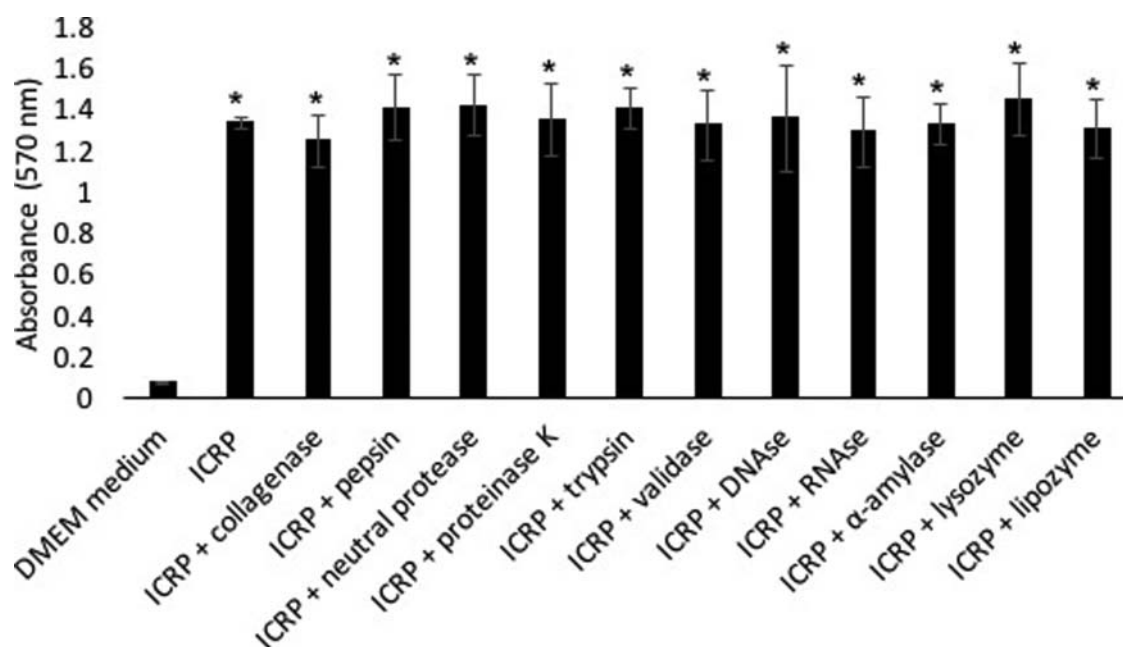

Figure 6. Antioxidant effect of IMMUNEPOTENT CRP exposed and not exposed to enzymatic treatment.
Note: Each sample was assayed in triplicate. Values are means from three independent experiments. * $P < 0.05$, significant difference (*post hoc* Dunnet test).
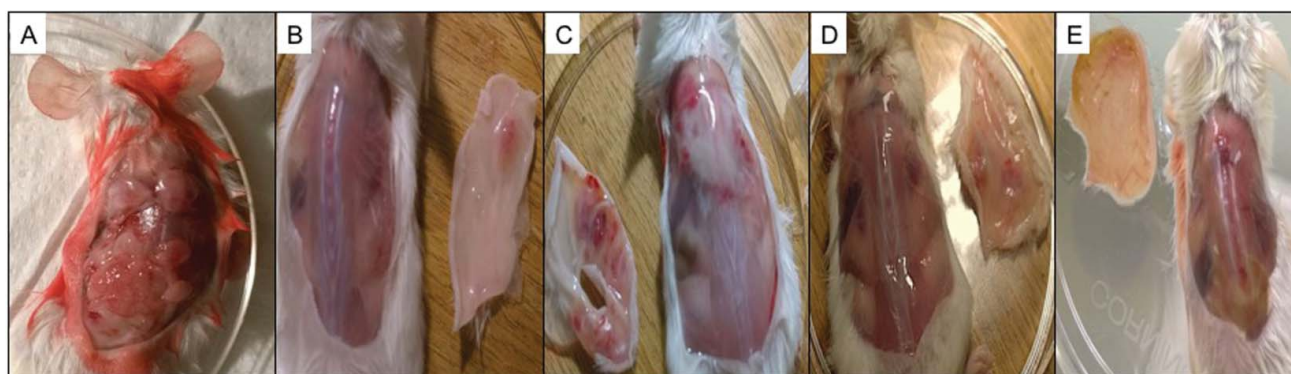
Figure 7. *In vivo* antitumour activity of IMMUNEPOTENT CRP and IMMUNEPOTENT CRP treated with proteinase K in BALB/c mice.
Note: Necropsy was performed after nine days daily treatment with PBS (A), 5-fu (20 mg/kg) (B), ICRP (3 units) (C), ICRP (5 units) (D) or ICRP treated with proteinase K (5 units) (E). The images show one representative mouse per group ($n = 3$).
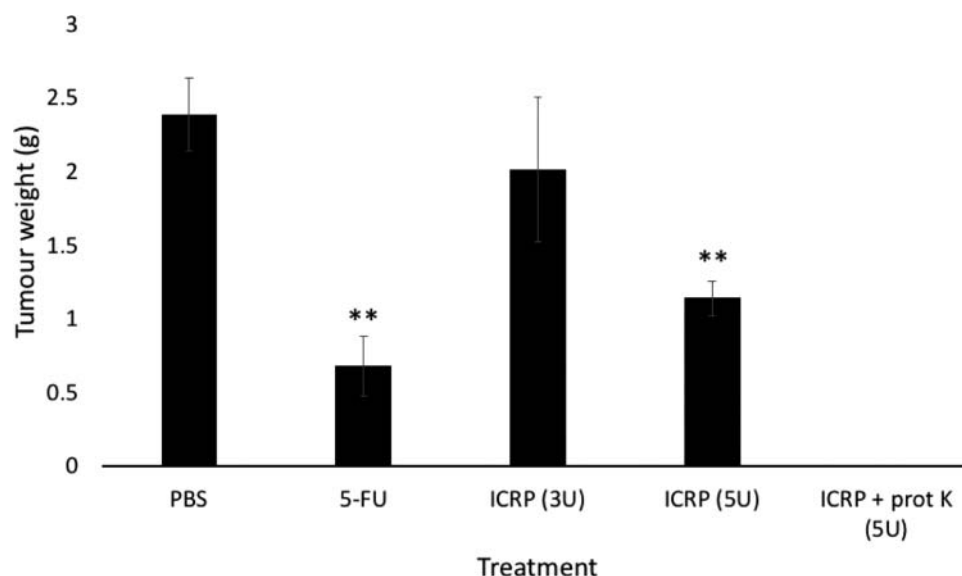
Figure 8. Tumour weight in BALB/c mice with murine lymphoma exposed to different treatments.
Note: PBS, 5-FU (20 mg/kg), ICRP (3 units), ICRP (5 units) or ICRP treated with proteinase K (5 units) were administered daily. On day 9 of therapy, the mice were sacrificed and the tumours were weighted. Data are mean values ($n = 3$). ** $P < 0.01$, significant difference (*post hoc* Dunnet test).

compared with PBS treatment. However, IMMUNEPO-TENT CRP (5 units dose) delayed tumour progression in a similar manner as the chemotherapeutic agent 5-FU, and IMMUNEPOTENT CRP treated with proteinase K (5 units dose) prevented the formation of any tumour foci during the evaluation period (Figures 7 and 8). In a previous report, we achieved similar results (a 50% tumour reduction) in a melanoma mice model using IMMUNE-POTENT CRP [21]. It would be interesting to corroborate the antitumour effect of IMMUNEPOTENT CRP treated with proteinase K on this model as well.

Conclusions

The results from this study showed that the antioxidant and antitumour biological activities of IMMUNEPOTENT CRP, are resistant to enzymatic treatment with proteases, nucleases, polysaccharide-degrading enzymes and lipases. Furthermore, IMMUNEPOTENT CRP treated with proteinase K can be used to improve and optimise our product formulation for cancer treatment. For this reason, more clinical studies should be performed.

Disclosure statement

The authors declare that they have no competing interests.

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