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Original Paper

Characterization and Industrial Application of the Enzyme Papain Through the Use of a Biosensor

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Key Words
Casein • Biosensor • Low environmental impact • Papain • Whole milk

Abstract
Background/Aims: Casein influences coagulation, yield, and product quality. This study aimed to develop and validate a papain (EC 3.4.22.2)-based biosensor to quantify casein in whole milk, evaluating its kinetic behavior and comparing it with HPLC. ***Methods:*** Papain was immobilized on nylon membranes and a cassava starch biopolymer. Enzyme kinetics (KM), stability, sensitivity, precision, and linearity of the system were evaluated. A t-test ($\alpha = 0.05$) was applied to compare the casein concentration obtained by the biosensor versus HPLC ($n = 20$). Interference from milk proteins and calcium was also analyzed. ***Results:*** The biosensor showed high affinity for casein (KM = 0.037 mM), with a linear range of 0.001–0.03 mM ($R^2 = 0.9974$) and a response time <5 s. No significant differences were found with HPLC ($p = 0.0665$). Stability reached 70 days at 4°C and was reusable up to 15 times. ***Conclusion:*** The developed biosensor proved to be an accurate, fast, and sustainable analytical alternative for the determination of casein in complex milk matrices.

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Introduction

The use of proteolytic enzymes is widespread, for example, in the medical field due to their ability to improve and prevent digestive disorders, mainly due to high protein intake, causing digestive discomfort (Rinninella et al, 2023). Other applications are due to their capacity to improve the assimilation of ingested proteins, resulting in high protein assimilation (Lee et al, 2023; Tornberg, 1996).

At the biochemical level, they are capable of reducing the activation energy necessary to initiate a reaction, acting as catalysts and maintaining the form during its transformation, allowing it to take place rapidly, without modification in its structure. In the food industry, their action has been positively proven, for example on the tenderness of meat, as well as in the production of cheese as a coagulant Mohd et al, 2023; Nicosia et al. 2022.

The enzyme activity depends directly on the extraction and purification treatments to which it is subjected, normally the crude extract is subjected to techniques to permeabilize the protein membranes present, allowing the exclusive passage of the enzymes, this stage can cause part of its initial activity to be lost; in a second treatment the enzymes are extracted using buffers or by freezing techniques, to bring them to a rapid thawing, thus allowing to break the cell walls, facilitating the extraction. To finish the process, the enzyme is purified; this mechanism is designed in a specific way, considering the physical and chemical properties with the objective of gradually separating the components considered not useful, thus improving the specific activity of the enzyme (Ghobadi et al, 2012).

Specifically, papain (EC 3.4.22.2) is a proteolytic enzyme whose structure consists of a polypeptide chain with three disulfide bridges and a sulfhydryl group necessary for proteolytic activity. It has a molecular weight of 23406 Dalton. This enzyme is very resistant to denaturation by the presence of high concentrations of chemicals due to the three calcium disulfides it possesses, these allow strong interactions between its side chains (Ayodipupo et al. 2023; Danait-Nabar et al, 2023). The functioning of this enzyme consists of attacking the carbon of the carbonyl group of the peptidic chain, achieving the breakdown of proteins due to its catalytic activity (Rajasekaran, et al. 2011). Due to this characteristic it is widely used in the industry as a clarifier in juices, in the dairy industry it is used to improve the proteolysis of mature cheese by increasing the concentration of free amino acids, in the meat industry to modify the texture of beef (Türkyılmaz et al, 2024; Oliveira Ribeiro et al, 2023; Li et al, 2022). The development of a sensor to determine the presence of casein may be beneficial because this parameter influences, for example, coagulation time, curd yield, and lower cheese yield (Faggion et al., 2025). Recent studies have also investigated the effect of using milk with high levels of casein on the properties of mozzarella cheese. It was observed that, at high concentrations, casein leads to an increase in the calcium content of the cheese. This parameter also influenced the moisture content of the finished product: the higher the casein concentration, the lower the moisture content. Finally, it had an effect on the sensory characteristics of the cheese, with instrumental hardness and sensory firmness being observed (Swaminathan et al, 2025). The use of infant formulas based on hydrolyzed proteins also has industrial applications, since when manufacturing these foods using casein hydrolysates, the degree of immunogenicity must be analyzed to assess their ability to induce an immune response in the body, identifying possible allergens, and also allowing for the optimization of the production process, as it is optimized by identifying the manufacturing conditions to minimize the formation of immunogenic peptides or proteins (Lindholm et al, 2024; Cui et al, 2023).

In this context, the use of a biosensor using papain as a biorecognition element can be useful for the food industry, facilitating the monitoring of this compound during production.

The detection mechanism is based on the transfer of electrons (potential variation) that occurs from the active center of papain to casein. This transfer of electrons can be considered

a redox reaction in which papain acts as a reducing agent. This is illustrated in the following diagram:

$$_{\text{reduced}}\text{papain} + \text{Casein} \qquad _{\text{oxidized}}\text{Papain} + \text{Small peptides} + \text{Amino acids (eq. 1)}$$

The detection mechanism for casein is shown in Fig 1.

There are different methods to quantify casein in foods. For example, in goat milk, protein fractions have been weighted by identifying genetic variants of caseins using Reverse-Phase High-Performance Liquid Chromatography (HPLC-UV) (Secchi et al, 2025). Casein determines the yield and affects the quality of cheese, so methods based on tryptophan emission spectra and casein content have been developed as a reference, achieving results in less time because long sample preparations are avoided (Ma et al, 2021)

It has been determined that casein is capable of forming hydrophobic interactions and disulfide bonds with insoluble dietary fibers to reduce the fermentation time during yogurt gelatinization. This new industrial application will reduce costs and increase the nutritional quality of this food matrix (Wang et al, 2025).

Quantification of this protein has been performed in different samples of interest (infant formulas and medical foods) by liquid chromatography-tandem mass spectrometry, due to the importance of casein in human well-being, specifically in patients suffering from phenylketonuria (Feng et al, 2023).

Therefore, the main objective of this study was to investigate the kinetic interaction between papain and the casein substrate in order to quantify this protein in whole milk using a biosensor.

Materials and Methods

Chemicals and Papain latex E.C:3.4.22.2 from Carica papaya, with each vial containing 29.6 mg of protein, casein sodium salt from bovine milk, were from Merk (Merck KGaA, Darmstadt, Germany), glutaraldehyde (50%), and the HPLC grade sulfuric acid was supplied by Sigma (Sigma -Aldrich, St Louis, MO, USA). The preactivated immunodyne ABC membrane (Nylon 6, 6 pore size 0.45 um) was supplied by Pall Europe (Porsmounth, UK). Sodium phosphate, anhydrous sodium acetate was obteined from Panreac (Panreac Química, Barcelona, Spain). Milli-Q deionized water was used throughout.

Preparation of the Papain

The enzyme was prepared by dissolving it in 10 mL of 5 mM buffer solution at pH 6.5 with an activity 10.5 U/mL aliquoted in 0.5mL Eppendorf tubes and frozen stored at -80ºC until use.

Equipment

A Dual Digital Model 20 amperometric sensor (Rank Brothers, Bottisham, Cambridge, England) with nylon membranes and a cassava-based biopolymer was used, the latter prepared as described by Anchundia et al, 2025. It consists of two electrodes, one working electrode and one reference electrode. The reference electrode is made of a silver ring (Ag/AgCl), while the working electrode is made of a platinum disc, which detects the potential variation during the enzymatic reaction (equation 1). These electrodes are connected to a transducer that amplifies the signal so that the information can be recorded and analyzed on a terminal (computer).

Chromatographic analysis was performed using YL9100 equipment manufactured by Lin Instrument CO, LTD (Korea).

Sample

In the first stage, it was necessary to isolate the casein. Due to the composition of milk, it was first necessary to separate the soluble salts from the casein. To do this, acid hydrolysis was carried out with 3 M hydrochloric acid, which was added drop by drop to coagulate the casein, adjusting the pH to 4.5. Next, ultracentrifugation was performed at 100, 000 x g, 15 °C, for 10 minutes using a Beckman Coulter

centrifuge (GmbH). The supernatant was filtered, separating the coagulated casein from the whey, and a white precipitate was obtained that was easy to handle.

In a second stage, the casein solution was prepared by weighing 250 mg of casein, adding 20 ml of distilled water and 5 ml of 5 N NaOH, and stirring until a total casein solution was obtained. This was frozen until use at -20 °C.

Biosensor preparation and operation

For the reaction to occur in the sensor it is necessary to adjust the electrical current of the circuit (-600mV) which must be closed by placing a few drops of KCl on the electrode and wait for it to stabilize. Next, the sensitivity of the sensor is adjusted by saturating the reaction cell with 100% oxygen. This stabilizes the base line, allowing the change in potential (mV/S) to be observed as the enzymatic reaction progresses. This value was found to be proportional to the concentration of casein. The identification process takes less than 5 seconds. The sensor's operating method has been described previously by Jadán et al, 2017.

The reaction cell was maintained at the optimum enzyme temperature (53ºC) by recirculating the water, previously heated with a thermostat.

Immobilized enzyme sensor

The percentage of glutaraldehyde needed to immobilize papain was studied for the two surfaces, ranging from 0.5% to 5% for the nylon membrane; and from 3% to 8% for the cassava-based biopolymer; the values were set at 2% and 6%, respectively.

Therefore, from a total mixture of 100 µL of solution, 60 µL of 5 mM sodium phosphate buffer, pH 6.5, with 10 µL of 2% and 6% glutaraldehyde and 30 µL of papain solution (12 U/mg) was poured onto the membranes (Nylon and Cassava Biopolymer, 1cm ^{2}), and allowed to dry for 1 hour before starting the immobilization process.

To avoid possible interference with food matrices, 5 µL of 2% cellulose acetate was added after drying (Qiong et al, 1998).

Description of the chromatographic

The analysis to quantify lysine in dairy product extracts and model systems was performed after derivatization with o-phthalaldehyde (OPA). A Rezex ROA-Organic Acid H+ thermostatic column (8µM x 300 x 7.8 mm) was used at 40°C, with sulfuric acid at a concentration of 0.02 N. The sample volume used was 20 µM x 300 x 7.8 mm. The sample volume used was 20 µL, the sample was diluted using sulfuric acid with a flow rate of 1mL/min in the mobile phase. Fluorescence detection used excitation and emission wavelengths of 230 nm and 450 nm, respectively.

Two solvents were used for the gradient: 20mM sodium acetate buffer, pH 7.2 (solvent A), and 100mM sodium acetate, pH 7.2/acetonitrile/methanol (30:35:35) v/v as solvent B. The mobile phase was bubble-free by degassing and filtration. The linear range used to quantify lysine was between 0.001 and 0.03 mM.

Use of the enzymatic sensor to analyze dairy samples

To analyze casein in pasteurized whole milk, the sample was taken from the local industry. The method was validated and its results were compared with HPLC.

Validation

Reproducibility was established by injecting (0.01 mM casein standards or dairy product samples) consecutively, 25 times into the immobilized enzyme system on the same day, using the same equipment and under the same conditions. The stability of the membrane stored at different temperatures was evaluated.

Linearity was achieved by analyzing triplicate patterns ranging from 0.001 to 0.03 mM for the immobilized enzyme system using the ordinary least squares (OLS) regression method. The sample dilution was 1/22 to obtain a strong signal, avoiding interference.

To determine the membrane's stability under operational conditions, successive injections of 0.01 mM casein were applied to a single membrane, and the response was recorded until 50% of its initial activity was observed. The results were expressed as function of time. The correlation, using different concentrations, between the sensor and HPLC were compared.

Statistical method. The increase and variation in casein concentrations were monitored using

Statgraphics Plus v5.1 software. To evaluate the linearity between the values obtained by the biosensor and the reference chromatographic method (HPLC), the coefficient of determination (R^2) and the standard error of the estimate (SE) were calculated. An R^2 value close to 1, accompanied by a low SE, indicates a robust linear relationship and good predictive capacity of the model, suggesting agreement between the two analytical methods.

In order to determine whether there were significant differences between the casein concentrations obtained by HPLC and those recorded by the biosensor, a t-test for two independent samples with bilateral contrast was applied. The null hypothesis (H_0) states that there are no statistical differences between the population means of both methods (H_0: μ_biosensor = μ_HPLC), while the alternative hypothesis (H_1) states that these means are significantly different (H_1: μ_biosensor ≠ μ_HPLC).

To evaluate the agreement between casein concentrations determined using the developed enzymatic biosensor and the reference chromatographic method (HPLC), a Student's t-test for paired samples was applied. A total of 20 independent samples were analyzed, each processed simultaneously by both methods. The concentrations obtained were expressed in millimoles (mM), and the corresponding means and standard deviations were calculated for each technique.

The analysis was performed at a significance level of α = 0.05. The t and p values were determined with 30 degrees of freedom, and the null hypothesis of equality of means between the two methods was evaluated. The difference between the mean values obtained by the biosensor ($\dot{X}$ = 1.30 mM, s = 0.1316) and by HPLC ($\dot{X}$ = 1.16 mM, s = 0.1452) was analyzed to determine whether it was statistically significant.

Results

Biosensor

During the reaction of the enzyme (peptidase C1) with the substrate, a redox reaction occurred that generated a potential difference at the baseline. This variation, detected by the sensor electrode, was directly proportional to the concentration of casein in the sample (whole milk) (equation 1). The least interference was observed at 5 seconds with a potential of -0.6 mV, eliminating noise. The biorecognition signal originated at the platinum base (1.5 mm in diameter) and was transmitted to the transducer, where it was amplified (Fig. 1). The coefficients of variation (CV) for whole milk and standards from consecutive injections were 3.15% and 1.7%, respectively. The optimum working pH was 6.5 (0.05 M sodium phosphate), and the temperature was 53ºC.

Biosensor features

Different concentrations of the substrate (casein) were prepared in ascending order. Substrate concentration was directly proportional to the signal up to 0.3 mM; above this, the velocity showed an asymptotic trend, consistent with active-site saturation (Fig. 2). Using

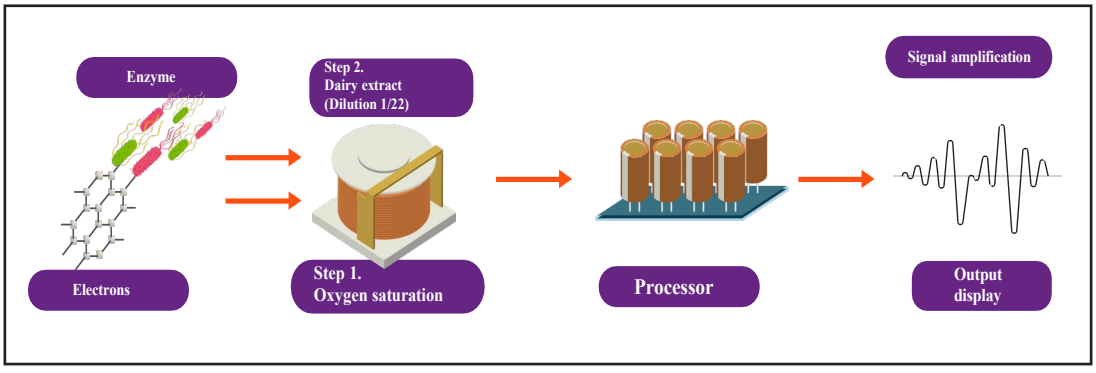


Fig. 1. Enzymatic mechanism and biosensor system for the detection of casein in dairy products.

this relationship and the Hanes–Woolf plot (Fig. 3), the Michaelis–Menten constant (KM) was determined, indicating high affinity of papain for the substrate (0.037 mM). The plot was constructed using:

$$S/v = S/Vmax + KM/Vmax \quad \text{(Eq. 2)}$$

The study was also carried out with the immobilized enzyme. Using a nylon membrane reduced activity by 33%, while the cassava biopolymer reduced activity by only 18%. Small reagent volumes produced clear amperometric signals: 30 µL enzyme, 6 µL sample (or standard), and 100 µL phosphate buffer; all assays used a 1/22 dilution (see also Jadán et al., 2023).

Fig. 2. Enzyme sensor response represented as the enzyme reaction rate as a function casein concentration (n=3).

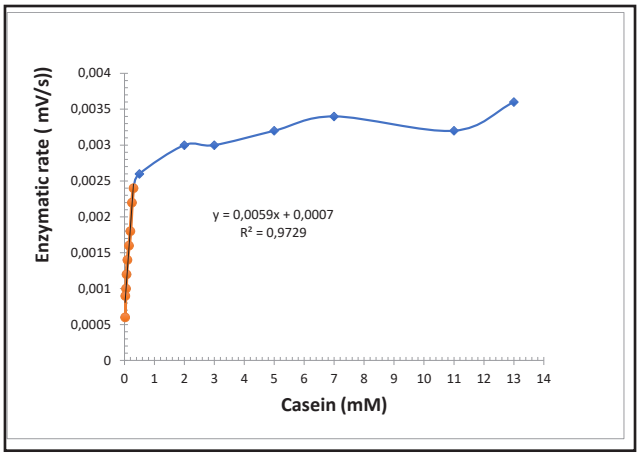
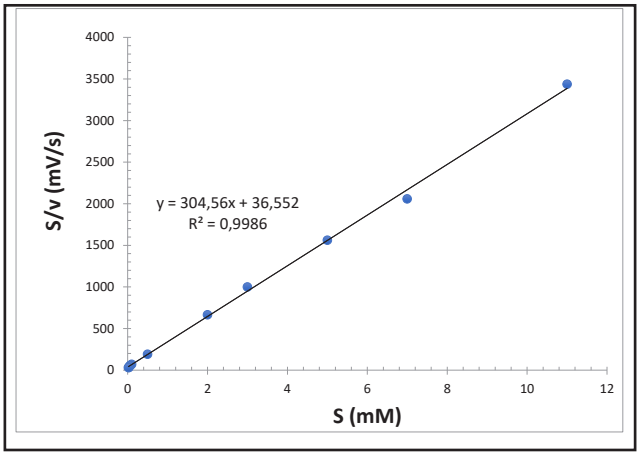
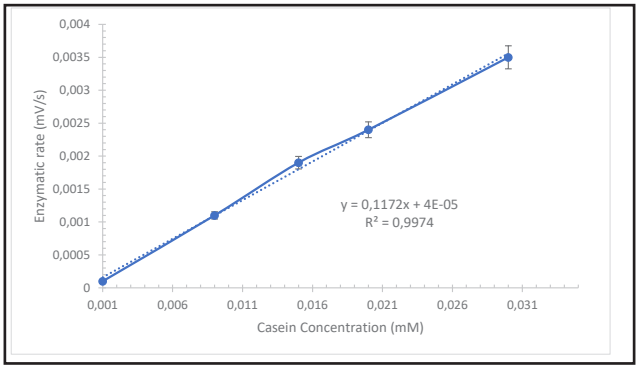


Fig. 3. Hanes-Woolf diagram. Graphic representation of the Kinetic parameters of the enzyme



papain**Fig. 4.** Calibration curve obtained with a biosensor Peptidase _C1 with enzyme immobilizated



Calibration curves

With the cassava biopolymer membrane, the linear range was 0.001–0.03 mM (R^2 = 0.9974). The detection limit was equivalent to 0.002 g (Fig. 4). With nylon, the linear range was 0.1–0.4 mM; the detection limit did not allow casein quantification in milk (data not shown). Repeatability (25 consecutive injections at 0.01 mM) yielded CVs of 0.9% (standards) and 2.3% (samples).

Casein measurements using the biosensor compared with HPLC measurements

Biosensor results showed a high correlation with HPLC (R^2 = 0.9971; standard error 0.016; Fig. 5, Table 1). A two-sample t-test (α = 0.05) indicated no significant difference between biosensor ($\dot{X}$ = 1.3 mM, s = 0.1316) and HPLC ($\dot{X}$ = 1.16 mM, s = 0.1452): t = 0.0653 (df = 30), p = 0.0665.

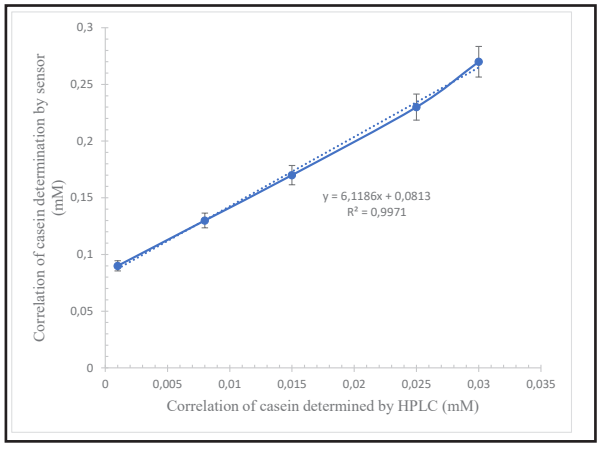


Fig. 5. Correlation between the content of casein obtained with the enzyme sensor with the immobilized enzyme and High Performance Liquid Chromatography (n=3).

Table 1. Measurements of casein levels by the enzyme sensor and High Performance Liquid Chromatography. Results are expressed in mM ± CV (n=3)

Sample (Pasteurized milk)	Enzyme Sensor (mM)	RSD (%)	HPLC (mM)	RSD (%)
1	0.035	3.2	0.032	0.4
2	0.031	1.1	0.033	0.3
3	0.035	3.2	0.031	1.6
4	0.032	3.1	0.035	0.9
5	0.034	1.5	0.037	2.3
6	0.037	2.2	0.039	3.2
7	0.033	2.3	0.031	2.5
8	0.031	1.2	0.034	1.8
9	0.038	3.2	0.035	0.7
10	0.031	2.1	0.033	0.4
11	0.035	2.4	0.031	2.3
12	0.032	1.9	0.036	1.1
13	0.034	3.1	0.031	1.3
14	0.031	1.8	0.033	0.6
15	0.035	3.5	0.037	1.3
16	0.038	2.1	0.033	0.7
17	0.031	1.4	0.033	0.4
18	0.034	2.1	0.036	0.4
19	0.031	1.9	0.035	1.4
20	0.038	1.7	0.037	0.3

Matrix Effects and Interferences

Potential interferents (lactoglobulin, lactalbumin, calcium) were assessed at 53°C using HPLC. Lactoglobulin showed the greatest interference (Table 2). Calcium added in equimolar amounts (0.01 mM) to casein produced only minor variations.

The stability of enzymes in storage

Papain immobilized on cassava biopolymer retained ~90% activity for up to 70 days at −4°C and could be reused up to 15 times. With nylon, ~90% activity persisted to day 40, declining to <10% by day 65 and disappearing by day 70 (Fig. 6).

Discussion

The papain-based biosensor quantified casein accurately and reproducibly under optimized conditions (pH 6.5, 53°C), producing electrochemical signals proportional to casein concentration. Kinetic analysis (Hanes–Woolf; Eq. 2) yielded a low KM (0.037

Table 2. Oxidation rate of the enzyme on selected compounds. Measured by HPLC and by the soluble enzyme biosensor. Values in percent (%)

Substrate	Oxidation rate (%) HPLC	Oxidation rate (%) Biosensor
Casein	100.0	100.0
Lactoglobulin	24.1	33.6
Lactalbumin	5.8	6.3
Calcium	1.7	0.9

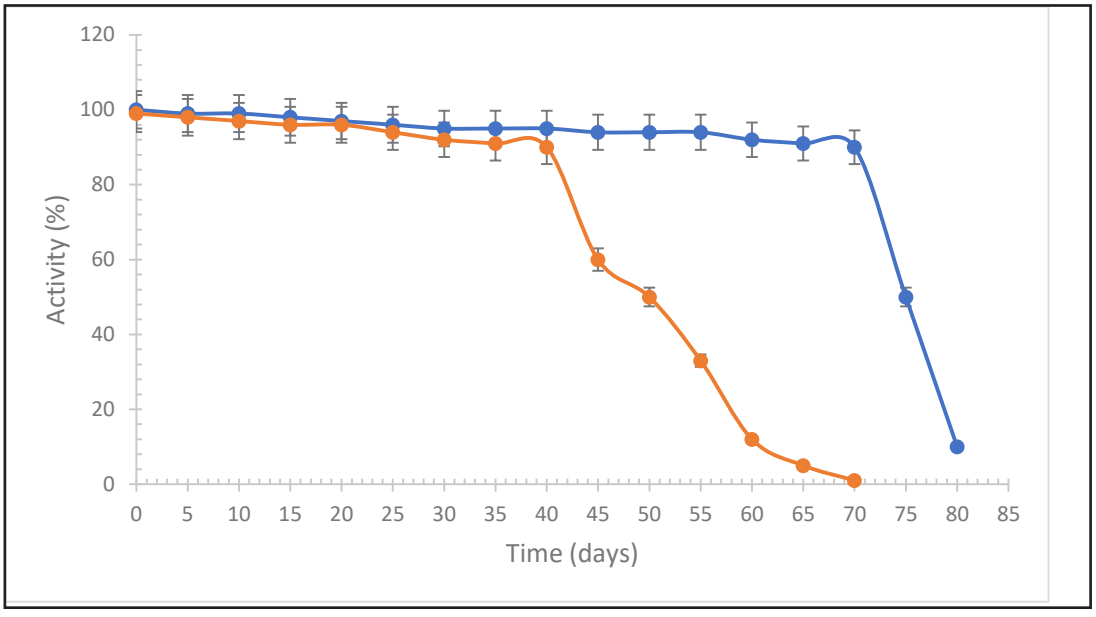


Fig. 6. Stability of papain in solution stored in refrigeration (4°C). n=3. The blue line represents the membrane with the cassava biopolymer, the orange line represents the nylon membrane.

mM), evidencing strong affinity. Immobilization on cassava biopolymer preserved activity and long-term stability better than nylon, likely due to favorable enzyme orientation and higher anchor density in the polysaccharide matrix. Analytical performance was strong (low detection limit, broad linearity, excellent repeatability), and agreement with HPLC was statistically indistinguishable, supporting the biosensor as a valid alternative for routine casein determination. Interference studies showed robustness to common milk constituents, with lactoglobulin being the main interferent and calcium effects minimal.

Conclusion

The method developed allowed low concentrations of casein to be quantified at low cost and with minimal detection time. The enzyme showed approximate stability of 70 days, allowing the enzyme immobilized with the cassava biopolymer to be reused up to 15 times. Stability has important industrial applications for increasing its use; future lines of research could include the use of new matrices for immobilization, such as other biodegradable polymers, as well as the application of cofactors in different proportions, which could further stabilize papain.

The method had a low environmental impact due to the small amounts of reagents used. During the operation time of the sensor the enzyme studied showed a high selectivity to the substrate (K_M=0.037 mM) so the industrial application of the enzyme is given as a new element for the biorecognition of this substrate. The results are comparable to those obtained by high performance liquid chromatography (HPLC).

In conclusion, it was possible to develop a reliable biosensor (R^2=0.9974), after studying the enzymatic kinetics, as well as its operating parameters linear range, limits of detection and quantification.

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Disclosure Statement

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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