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Sustainable Recovery of Actinidin From *Actinidia deliciosa* Peel Using Biocompatible Cholinium-Based Ionic Liquids

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ABSTRACT

Food waste valorization has emerged as an important strategy to recover high-value biomolecules from underutilized agro-industrial waste. Among them, actinidin, the major protease of *Actinidia deliciosa*, has attracted considerable interest due to its broad proteolytic activity and potential applications in food processing, nutrition, and biotechnology. In this work, actinidin was extracted from kiwifruit peel using aqueous solutions of cholinium-based ionic liquids (ILs). An initial solvent screening identified cholinium bitartrate [N₁₁₁(2OH)][Bit] as the most effective extraction medium. Molecular docking studies revealed that the interactions between actinidin and IL ions depend strongly on ion identity, particularly the anion. Process optimization identified 500 mM [N₁₁₁(2OH)][Bit], a solid-to-liquid ratio of 1:10 w/w, 600 rpm, 45°C, and 30 min as the most favorable extraction conditions, yielding a proteolytic activity of 5.388 U L⁻¹, a protein concentration of 1115.3 mg L⁻¹, and a specific proteolytic activity of 0.00483 U mg⁻¹ protein. The process achieved a Path2Green score of 0.392, indicating moderate sustainability potential, and a process for IL recyclability was proposed. These results support the potential of aqueous cholinium-based ILs for the valorization of kiwifruit peel while defining solvent recovery, toxicological assessment, and technoeconomic evaluation as priorities for further process development.

1 | Introduction

Food loss and waste have emerged as critical global challenges, driven by population growth and inefficiencies across food supply chains [1]. In the European Union, food waste reached 59.2 million tons in 2024, with Portugal generating approximately 2 million tons of food waste [2]. In this context, the valorization of food waste through the recovery of high-value compounds has emerged as a promising approach to support circular economy models, enhance resource efficiency, and contribute to the achievement of the United Nations Sustainable Development Goals, particularly SDG 12 (Responsible Consumption and Production), SDG 9 (Industry, Innovation, and Infrastructure), and SDG 13 (Climate Action) [3, 4].

Kiwifruit (*Actinidia deliciosa*) has gained significant importance in global fruit production due to its nutritional value, consumer acceptance, and adaptability to diverse climatic conditions [5, 6]. Nevertheless, its industrial processing generates significant amounts of waste (e.g., peels and seeds) [5, 7]. Despite being underutilized, these wastes are rich in bioactive compounds, including proteins, fibers, phenolic compounds, and digestive enzymes [6, 8]. Among these, actions are the major endogenous enzyme, accounting for approximately 50%–60% of the total soluble protein fraction [9]. This protease belongs to the cysteine protease family and has attracted considerable interest due to its broad proteolytic activity under mild conditions. Actinidin has been investigated for various applications, particularly in the food industry as a meat tenderizer and milk coagulant, and in

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nutritional and health-related formulations to improve the digestion of dietary proteins. In addition, its enzymatic properties also show potential for broader biotechnological applications involving protein hydrolysis and modification in pharmaceutical products, such as healing creams [10, 11]. These features position actinidin as a particularly attractive target biomolecule for recovery from kiwifruit waste [5].

Despite the advantages associated with protein recovery from plant biomass, extracting proteins from such complex matrices remains a significant challenge. As a result, protein extraction is still largely based on conventional media, including acidic or alkaline aqueous solutions and organic solvents [12]. However, these approaches often suffer from important drawbacks, such as long processing times, high solvent and energy consumption, low selectivity, and the possibility of protein denaturation or loss of activity [13]. This issue becomes even more critical when the target compound is an active enzyme, whose successful recovery depends on its removal from the biomass, the preservation of its structural integrity, and its catalytic function [14]. In this regard, ionic liquids (ILs) have emerged as promising alternative extraction media. Their unique and tunable physicochemical properties, such as negligible vapor pressure, adjustable polarity, and the ability to establish specific interactions (e.g., hydrogen bonding and electrostatic interactions), enable enhanced solvation and stabilization of biomolecules [12, 15].

ILs are salts composed of organic or inorganic ions, thus displaying lower melting temperatures than inorganic salts. Their physicochemical properties can be tailored through the selection of cation–anion combinations, enabling the design of task-specific solvents [16, 17]. Among them, cholinium-based ILs have gained attention due to their biocompatibility, relatively low toxicity, and environmental friendliness [18]. Importantly, these ILs have demonstrated the ability to preserve protein structural integrity and enzymatic activity, making them suitable for protein-related applications [19].

In this context, the present study evaluates the feasibility of recovering actinidin from kiwifruit peels using aqueous solutions of cholinium-based ILs as alternative extraction media. A series of cholinium-based ILs was systematically screened to evaluate proteolytic activity, total protein recovery, and specific proteolytic activity. In addition, molecular docking studies were conducted to provide insight into the interactions between actinidin and IL ions at the molecular level, as well as their potential roles in enzyme extraction and stabilization. The most promising IL was subsequently selected for process optimization, and the effects of extraction time, stirring speed, temperature, IL concentration, and solid-to-liquid ratio were evaluated, with the purpose of evaluating IL concentration in protein recovery and specific proteolytic activity.

2 | Materials and Methods

2.1 | Materials

Kiwifruit was purchased from a local supermarket in Aveiro, Portugal. The ILs cholinium acetate ($[N_{111}(2OH)][C_1CO_2]$, 98 wt%),

cholinium dihydrogen phosphate ($[N_{111}(2OH)][DHP]$, >98 wt%), cholinium dihydrogen carbonate ($[N_{111}(2OH)][DHC]$, >97), and cholinium bis(trifluoromethylsulfonyl)imide ($[N_{111}(2OH)][NTf_2]$, 99 wt%) were purchased from IOLITEC. Cholinium bromide ($[N_{111}(2OH)]Br$, >98 wt%) was acquired from TCI, while cholinium bitartrate ($[N_{111}(2OH)][Bit]$, 97 wt%) and cholinium chloride ($[N_{111}(2OH)]Cl$, 98 wt%) were purchased from Acros Organics.

Chemical reagents used to determine actinidin proteolytic activity, including azocasein, sodium acetate trihydrate (Scharlab), acetic acid, trichloroacetic acid (TCA), and potassium hydroxide, were obtained from Sigma–Aldrich unless otherwise specified. Dithiothreitol (DTT, 99 wt%, Acros Organics), sodium dodecyl sulfate (SDS, 99 wt%, Acros Organics), glycerol (99 wt%, Acros Organics), bromophenol blue (Merck), Novex Tricine SDS Running Buffer (Invitrogen, Thermo Fisher Scientific), Novex 16% Tricine Gel, 15 wells (Invitrogen, Thermo Fisher Scientific), NZYTech Protein Marker I (NZYTech), and BlueSafe (NZYTech) were used for sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE).

2.2 | Kiwifruit Peel Preparation

Kiwifruit was washed with soapy water and manually peeled. The peels were dried in a convection oven at 45°C for 48 h. The dried biomass was then ground using a mortar and pestle under liquid nitrogen to obtain a homogeneous powder. To minimize sample variability while preserving protein stability, the processed biomass was stored at 4°C and used throughout all experiments.

2.3 | Actinidin Extraction From Kiwifruits Peels Using Aqueous Solution of ILs

The solid–liquid extraction of actinidin from kiwifruit peels was performed using a Radleys Tech Carousel 12 Plus Reaction Station (Saffron Walden, Essex, UK), which enabled precise control of stirring speed and temperature. Preliminary extraction screening was carried out at a solid-to-liquid ratio of 1:10 w/w, under stirring at 400 rpm and 25°C for 30 min. To evaluate the performance of different ILs in actinidin extraction, aqueous solutions containing 50 mM of $[N_{111}(2OH)][C_1CO_2]$, $[N_{111}(2OH)]Br$, $[N_{111}(2OH)][Bit]$, $[N_{111}(2OH)]Cl$, $[N_{111}(2OH)][DHP]$, $[N_{111}(2OH)][Bic]$, or $[N_{111}(2OH)][NTf_2]$ were prepared in phosphate buffer at pH 7.4. Then, an optimization stage was performed using the most promising IL, $[N_{111}(2OH)][Bit]$, by evaluating the effects of extraction time (5–90 min), stirring speed (100–800 rpm), temperature (20–55°C), IL concentration (25–1000 mM), and solid-to-liquid ratio (1:10–1:50 w/w) on extraction performance. After extraction, the samples were centrifuged at 4000 rpm for 20 min using a Centrifuge 5804 (Eppendorf, Hamburg, Germany).

2.4 | Proteolytic Activity

The proteolytic activity of the protein-enriched supernatant was determined based on azocasein hydrolysis. To determine activity, a 0.5% (w/v) azocasein solution was prepared in 50 mM acetate buffer at pH 5.0. Then, 1 mL of the substrate solution was mixed

with 1 mL of the enzyme extract and incubated for 40 min at 25°C. After incubation, 1 mL of 15% (w/v) TCA was added to stop the reaction, and the mixture was centrifuged at 4000 rpm for 15 min. Subsequently, 2 mL of the resulting supernatant was mixed with 2 mL of 5 M KOH, and the absorbance was measured at 428 nm [20]. Proteolytic activity (A) was calculated according to Equation (1). One unit of activity was defined as the amount of enzyme required to produce an increase of 0.01 absorbance units at 428 nm per min, relative to the blank, under the assay conditions, and the results were expressed as U L^{-1} . All measurements were performed in triplicate. The corresponding blank samples were prepared using the same extraction medium, including the aqueous IL solutions, allowing the measured activity to be attributed only to the azocasein hydrolysis by the extracted protease.

$$A = \frac{\text{Abs}_{\text{sample}} - \text{Abs}_{\text{blank}}}{(0.01) * (\Delta t) * V_s} \quad (1)$$

where Abs is the absorbance; Δt is the analysis time (in min); V_s is the volume of the enzymatic solution (0.001 L).

2.5 | Protein Quantification and Specific Activity

Total protein content in kiwifruit extracts was determined according to the Bradford method [21]. Analyses were performed in 96-well microplates using 190 μL of previously prepared Bradford reagent and 10 μL of kiwifruit extract. After 5 min, absorbance was measured at 595 nm using a spectrophotometer. Quantification was performed using a standard curve constructed from a standard solution with 0–800 μL of bovine serum albumin (BSA). Results were expressed in mg protein L^{-1} . Specific proteolytic activity was calculated by normalizing the proteolytic activity of each extract to its total protein concentration, according to Equation (2), and protein recovery yield was calculated according to Equation (3):

$$\text{SA} = \frac{A}{C_p} \quad (2)$$

$$Y_p = \frac{C_p * V}{m_B} \quad (3)$$

where SA is the specific proteolytic activity (U mg^{-1} protein), A is the proteolytic activity (U L^{-1}), and C_p is the total protein concentration (mg L^{-1}). Y_p is the protein yield (mg g^{-1} biomass), V is the volume of the extraction medium (L), and m_B is the mass of dry kiwifruit peel used in the extraction (g).

2.6 | Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis (SDS-Page)

The protein profiles of the supernatants were analyzed by SDS-PAGE. Prior to electrophoresis, the samples were mixed at a 1:1 (v/v) ratio with sample buffer containing 4% (w/v) SDS, 20% (w/v) glycerol, 120 mM Tris–HCl (pH 6.8), 0.02% (w/v) bromophenol blue, and 31 mg of DTT. The mixtures were then denatured at 95°C for 5 min. The NZY Color Protein Marker (5 to 245 kDa) and the samples were loaded onto the polyacrylamide gels and run at 80 V for 1 h, followed by 1 h at 120 V. After electrophoretic

separation, the gels were stained with BlueSafe overnight at room temperature.

2.7 | Molecular Docking

The three-dimensional structure of actinidin (PDB ID: 2act) was retrieved from the Protein Data Bank (PDB). To prepare the protein structure for computational analysis, the protonation states of titratable residues were assigned using the Protein Prepare tool available on the PlayMolecule web server (playmolecule.org). This preparation was carried out at pH 7.4, and all crystallographic water molecules, ions, and cocrystallized ligands were removed from the original PDB file prior to docking calculations. The chemical structures of the ligands investigated in this study were built and optimized using Discovery Studio v21 (Accelrys, San Diego, CA, USA) [22]. Three-dimensional coordinates were generated and subsequently minimized using the Chem3D-MM2 protocol. The resulting ligand structures were then converted into .pdbqt format using AutoDockTools (ADT), with all rotatable bonds defined as active torsions whenever applicable. The prepared actinidin structure was also converted into .pdbqt format using the same software [23].

Docking simulations were performed using a grid box centered at the active site region of actinidin, including the catalytic residues typically associated with its proteolytic activity. The grid center coordinates (x , y , and z) were set to 1.484, 0.507, and 1.649, respectively, and the grid box dimensions were defined as 80 Å $\times$ 80 Å $\times$ 90 Å in order to encompass the binding pocket. The docking calculations were carried out using an exhaustiveness value of 100, num_modes = 10, and seed = 100. For each ligand, the binding pose with the lowest docking score was selected for further analysis.

3 | Results and Discussion

The recovery of actinidin from kiwifruit (*A. deliciosa*) peels was evaluated under various extraction conditions to identify the parameters that most strongly influence its activity. As an initial screening, the effects of IL type ($[\text{N}_{111}(\text{2OH})][\text{NTf}_2]$, $[\text{N}_{111}(\text{2OH})]\text{Br}$, $[\text{N}_{111}(\text{2OH})]\text{Cl}$, $[\text{N}_{111}(\text{2OH})][\text{Bic}]$, $[\text{N}_{111}(\text{2OH})][\text{DHP}]$, $[\text{N}_{111}(\text{2OH})][\text{C}_1\text{CO}_2]$, and $[\text{N}_{111}(\text{2OH})][\text{Bit}]$) were investigated in order to maximize the recovery of catalytically active actinidin under moderate and more sustainable extraction conditions. Cholinium-based ILs (50 mM) were investigated at a solid–liquid ratio of 1:10 (w/w), at 25°C for 30 min under stirring at 400 rpm. The pH was maintained at neutral levels using a phosphate buffer (pH 7.4) to provide a stable chemical environment for the comparative evaluation of the ILs and the phosphate buffer control. Previous studies [5] have shown that sodium phosphate at pH 7.0 is an effective medium for the extraction of actinidin, exhibiting higher specific activity than that obtained under various acidic and alkaline conditions. The results obtained are presented in Figure 1a and Table 1.

As all the investigated ILs share the same cation ($[\text{N}_{111}(\text{2OH})]^+$), the differences observed in actinidin extraction performance can be mainly attributed to the nature of the anion. In this context, the anion likely affects the ability of the aqueous IL medium

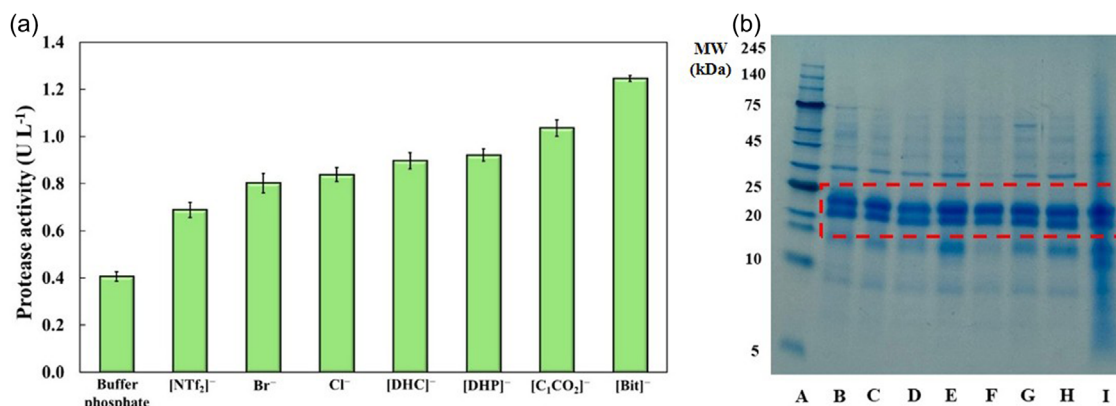


FIGURE 1 | (a) Proteolytic activity (U L⁻¹) of actinidin extracted from kiwifruit (*A. deliciosa*) using aqueous solutions of cholinium-based ILs (50 mM), with phosphate buffer as the control extraction medium. Extraction conditions: 30 min, 400 rpm, 25°C, and solid-to-liquid ratio of 1:10 (w/w); (b) SDS-PAGE of protein marker (A) and protease samples recovered using different cholinium-based ILs: [N₁₁₁(2OH)]Br (B), [N₁₁₁(2OH)][NTf₂] (C), [N₁₁₁(2OH)][DHC] (D), [N₁₁₁(2OH)]Cl (E), [N₁₁₁(2OH)][Bit] (F), [N₁₁₁(2OH)][DHP] (G), [N₁₁₁(2OH)][C₁CO₂] (H), and buffer control (I).

TABLE 1 | Proteolytic activity, total protein concentration, specific proteolytic activity, and protein yield obtained after the extraction of kiwifruit (*Actinidia deliciosa*) peel using phosphate buffer and aqueous cholinium-based IL solutions. Extraction conditions: 50 mM IL, solid-to-liquid ratio of 1:10 (w/w), 400 rpm, 25°C, and 30 min.

Extraction medium	Protease activity, U L ⁻¹	Protein, mg L ⁻¹	Specific activity, U mg ⁻¹	Protein yield, mg g ⁻¹ biomass
Buffer phosphate	0.405 ± 0.021	176.8 ± 4.8	0.00229 ± 0.00171	1.77 ± 0.04
[N ₁₁₁ (2OH)][NTf ₂]	0.688 ± 0.032	304.3 ± 5.5	0.00226 ± 0.00169	3.04 ± 0.09
[N ₁₁₁ (2OH)]Br	0.801 ± 0.041	329.0 ± 3.7	0.00244 ± 0.00155	3.29 ± 0.08
[N ₁₁₁ (2OH)]Cl	0.837 ± 0.030	336.5 ± 3.5	0.00249 ± 0.00161	3.37 ± 0.11
[N ₁₁₁ (2OH)][DHC]	0.896 ± 0.034	343.4 ± 3.9	0.00261 ± 0.00152	3.43 ± 0.08
[N ₁₁₁ (2OH)][DHP]	0.920 ± 0.026	352.6 ± 5.3	0.00261 ± 0.00149	3.53 ± 0.08
[N ₁₁₁ (2OH)][C ₁ CO ₂]	1.035 ± 0.034	369.5 ± 4.7	0.00280 ± 0.00156	3.70 ± 0.10
[N ₁₁₁ (2OH)][Bit]	1.245 ± 0.012	404.5 ± 4.4	0.00308 ± 0.00160	4.05 ± 0.09

to disrupt non-covalent interactions between actinidin and the biomass components, enhance protein solubilization, and promote the migration of actinidin from the kiwifruit peel matrix to the liquid phase. Therefore, the capacity of each anion to establish favorable interactions with water, biomass components, and the protein microenvironment appears to play a central role in determining the final proteolytic activity measured in the extract immediately after processing. Since protein extraction in IL-based systems depends strongly on the balance between protein-water and protein-IL interactions, the biocompatibility of the anion becomes an important factor in protein recovery [24, 25]. In this context, the anion likely affects both the extraction of actinidin from the kiwifruit peel matrix and the proteolytic activity measured in the resulting extract. Since protein extraction and activity in IL-based systems depend strongly on the balance between protein-water and protein-IL interactions, the biocompatibility of the anion becomes a key factor in determining protein recovery. The results obtained indicate that ILs containing anions with higher hydrogen-bond basicity, particularly [N₁₁₁(2OH)][Bit] and [N₁₁₁(2OH)][C₁CO₂], are associated with higher proteolytic activities and better extraction performance than those containing weakly coordinating or more disruptive anions, such as [N₁₁₁(2OH)][NTf₂] [24].

Although hydrophobic ILs are often described as favorable media for protein-related applications, this trend was not reflected in the extraction results obtained here. In the case of [N₁₁₁(2OH)][NTf₂], the lower proteolytic activity observed after extraction may indicate that this IL was less effective in promoting the recovery of catalytically active actinidin from the kiwifruit peel matrix. This behavior may be associated with the bulky and weakly hydrated nature of the [NTf₂]⁻ anion, which can affect solvent-biomass and solvent-protein interactions, thereby limiting solubilization and transfer of actinidin to the liquid phase [25].

Among the ILs studied, the proteolytic activity extracted followed the order: [N₁₁₁(2OH)][Bit] (1.245 ± 0.012 U L⁻¹) > [N₁₁₁(2OH)][C₁CO₂] (1.035 ± 0.034 U L⁻¹) > [N₁₁₁(2OH)][DHP] (0.920 ± 0.026 U L⁻¹) > [N₁₁₁(2OH)][DHC] (0.896 ± 0.034 U L⁻¹) > [N₁₁₁(2OH)]Cl (0.837 ± 0.030 U L⁻¹) > [N₁₁₁(2OH)]Br (0.801 ± 0.041 U L⁻¹) > [N₁₁₁(2OH)][NTf₂] (0.688 ± 0.032 U L⁻¹). Proteolytic activity was analyzed in relation to total protein concentration in order to determine whether differences in the increase in protein recovery were due to changes in catalytic activity. Total protein concentration increased from 176.8 mg L⁻¹ in the phosphate-buffer control to 384.5 mg L⁻¹ in the [N₁₁₁(2OH)][Bit] extract and the protein yield increased from 1.77 to 3.85 mg g⁻¹

biomass. Thus, $[N_{111(2OH)}][Bit]$ increased total protein recovery by approximately 2.17-fold compared with phosphate buffer. In contrast, proteolytic activity increased from 0.405 to 1.245 U L⁻¹, corresponding to a 3.07-fold enhancement. The greater relative increase in catalytic activity than in total protein recovery resulted in an increase in specific proteolytic activity from 0.00229 U mg⁻¹ protein in the buffer extract to 0.00324 U mg⁻¹ protein in the $[N_{111(2OH)}][Bit]$ system, reflecting an increase of approximately 41%. Proteolytic activity reflects the combined effects of protein recovery and preservation of catalytic functionality and, therefore, should be interpreted in relation to total protein concentration and specific activity. The increase in total protein yield observed for $[N_{111(2OH)}][Bit]$ was accompanied by a proportionally greater increase in proteolytic activity, resulting in the highest specific proteolytic activity among the evaluated extraction media. These data show that the superior performance of the bitartrate-based IL resulted in higher protein solubilization and a better catalyst recovery of protease per unit of total extracted protein. The simultaneous increase in protein yield and specific activity therefore supports the ability of $[N_{111(2OH)}][Bit]$ to provide extraction conditions that favor both protein recovery and retention of proteolytic functionality.

The experimental differences among the evaluated ILs are consistent with the concept of specific ion effects described by the Hofmeister series, in which anions generally exert a stronger influence on protein hydration, stability, and activity than cations. However, the behavior of proteins in aqueous IL solutions cannot be represented by a universal Hofmeister sequence, since the magnitude and even the direction of stabilization or destabilization may depend on the protein structure, IL cation–anion combination, concentration, and solution conditions [26]. Previous studies [27–29] have shown that strongly hydrated anions can favor preservation of protein structure in several IL-containing systems, whereas weaker hydrated and hydrophobic ions may promote destabilization through direct ion–protein interactions and perturbation of the hydration shell. Nevertheless, deviations from classical Hofmeister ordering have also been reported, emphasizing that direct ion–protein interactions must be considered in addition to bulk water structuring effects. In the present study, the higher proteolytic activity and specific activity observed for $[N_{111(2OH)}][Bit]$, $[N_{111(2OH)}][C_1CO_2]$, and $[N_{111(2OH)}][DHP]$, compared with the halide- and $[NTf_2]$ -containing systems, are therefore interpreted as an ion-specific response rather than as strict adherence to a classical Hofmeister sequence.

In agreement with this interpretation, Feitosa et al. [13] reported that $[N_{111(2OH)}][Bit]$ enhanced protein extraction from Brazilwood seeds, attributing this effect to its oxygen-rich structure and corresponding ability to establish hydrogen-bonding interactions with water, thereby improving solvation capacity. In contrast, chloride and bromide anions are generally found near the transition between kosmotropic and chaotropic ions, which may explain their comparatively lower performance. These anions interact less favorably with the protein's hydration network. Thus, the lower extraction performance observed for $[N_{111(2OH)}]Cl$ and $[N_{111(2OH)}]Br$ likely reflects a less favorable balance among water–anion, protein–water, and protein–IL interactions. Valente et al. [30] observed that cholinium-based ILs were more suitable media for RuBisCO extraction from spinach leaves than several other IL classes, demonstrating that the chemical structure of the ions

strongly affects both extraction performance and protein stability. In contrast, imidazolium-based ILs, although capable of extracting the target protein in some cases, were more strongly associated with protein aggregation and potential denaturation. Importantly, the total protein and specific activity data provide additional experimental support for this interpretation. Although all IL generally increased total protein recovery relative to phosphate buffer, the magnitude of the increase in specific proteolytic activity depended on the anion. For example, $[N_{111(2OH)}][NTf_2]$ increased protein concentration from 176.8 to 304.3 mg L⁻¹ but displayed a specific activity (0.00226 U mg⁻¹) comparable to that of the phosphate control (0.00229 U mg⁻¹). This result indicates that the higher proteolytic activity obtained with $[N_{111(2OH)}][NTf_2]$ predominantly reflected higher protein solubilization rather than an increase in catalytically active protease. In contrast, $[N_{111(2OH)}][C_1CO_2]$ and particularly $[N_{111(2OH)}][Bit]$ exhibited higher specific activities of 0.00285 and 0.00324 U mg⁻¹, respectively, demonstrating that the identity of the anion influenced protein recovery and the catalytic quality of the recovered protein fraction.

Compared with the phosphate-buffer control, all IL-containing media increased the amount of protein recovered in the liquid phase. The buffer extract contained 176.8 mg L⁻¹ protein, with a protein yield of 1.77 mg g⁻¹ biomass, whereas the IL-containing systems yielded between 304.3 and 384.5 mg L⁻¹ protein (3.04–3.85 mg g⁻¹ biomass). However, the increase in total protein recovery was not proportional to the increase in proteolytic activity for all ILs. For example, $[N_{111(2OH)}][NTf_2]$ increased protein concentration to 304.3 mg L⁻¹, representing an approximately 72% increase relative to phosphate buffer, whereas its specific proteolytic activity remained essentially unchanged (0.00226 versus 0.00229 U mg⁻¹ protein). This behavior shows that $[N_{111(2OH)}][NTf_2]$ enhanced the solubilization of the protein fraction without increasing proteolytic activity per unit of recovered protein. A distinct result was observed for $[N_{111(2OH)}][Bit]$, for which protein recovery increased by approximately 117%, while volumetric proteolytic activity increased by approximately 207%. Consequently, the specific activity reached 0.00324 U mg⁻¹ protein, approximately 41% higher than that of the phosphate-buffered extract. These different behaviors demonstrate that the chemical nature of the IL influences the extent of protein recovery and the proteolytic activity of the recovered protein fraction [30].

After the solid–liquid extraction using all ILs, the extracts containing actinidin protease were analyzed by SDS–PAGE. From Figure 1b, it is possible to observe a protein band consistently at approximately ≈24 kDa for all actinidin-recovered samples using different cholinium-based ILs, in good agreement with the reported molecular mass [31]. The molecular weight of actinidin samples obtained with different extraction conditions (e.g., solid-to-liquid ratio, temperature, and time) was also analyzed by SDS–PAGE, showing the same band around ≈24 kDa (cf. Figure S1). The recurrent presence of this band across the different extracts supports the interpretation that the optimized cholinium-based IL systems were able to recover the target protease from kiwifruit peel while maintaining it in a soluble form suitable for electrophoretic analysis.

In the initial IL screening, a band between 20 and 25 kDa was observed for all cholinium-based ILs, in agreement with the actinidin molecular weight (≈24 kDa). Differences in band

intensity qualitatively followed the proteolytic activity results, with $[N_{111(2OH)}][\text{Bit}]$ and $[N_{111(2OH)}][C_1CO_2]$ showing the strongest profiles, which agrees with the proteolytic activity shown in Figure 1. Although the buffer control also displayed a band in the same region, its lower enzymatic activity indicates that IL-containing media provided a more favorable environment for the recovery of catalytically active actinidin. The presence of additional bands confirms that proteins other than actinidin were coextracted from the kiwifruit peel matrix. Therefore, SDS-PAGE alone does not demonstrate selective actinidin extraction. Nevertheless, the comparatively cleaner electrophoretic profile obtained with $[N_{111(2OH)}][\text{Bit}]$ is consistent with the higher specific proteolytic activity measured for this extract. The protein quantification and activity data therefore support the recovery of a protein fraction with greater proteolytic activity, rather than the purification of actinidin as an individual protein species. The presence of additional bands confirms that proteins other than actinidin were coextracted from the kiwifruit peel matrix. Therefore, the electrophoretic profiles were interpreted qualitatively by visual inspection. Nevertheless, the extract obtained with $[N_{111(2OH)}][\text{Bit}]$ displayed a comparatively cleaner electrophoretic profile and the highest specific proteolytic activity among the evaluated systems. These observations are consistent with a greater relative enrichment of the proteolytically active protein fraction.

The presence of additional bands in the SDS-PAGE profiles indicates that proteins other than actinidin were coextracted from the kiwifruit peel matrix, which is expected in a direct extraction from plant biomass without a dedicated purification step. In this study, the aim was to recover an enzymatically active extract under moderate and more sustainable conditions. This is similar to previous studies showing that crude or partially purified kiwifruit extracts can be directly applied in food systems, such as milk clotting and meat tenderization, without requiring a highly purified enzyme preparation [31, 32]. In this sense, avoiding extensive purification steps may represent an important advantage, as it reduces solvent, energy, and material consumption, contributing to a lower-cost, more sustainable valorization route for kiwifruit peel. Dhiman et al. [33] reported that crude actinidin obtained from *Actinidia deliciosa* pulp exhibited an activity of 59.67 U mL^{-1} using 100 mM sodium phosphate buffer, while Katsaros et al. [34] found a concentration of 1.35 U L^{-1} in kiwi pulp that had simply been crushed and centrifuged. Although these values cannot be directly compared to the proteolytic activities obtained in this study due to methodological differences, the results support the idea that the concentration of actinidin is higher in kiwifruit pulp than in extracts from the peel [35]. Notably, the present study demonstrated that kiwifruit peel, despite being a waste and likely containing lower enzyme levels than the pulp, remains a relevant and technically viable source of active actinidin when an appropriate extraction medium is employed. Thus, the recovery of this protease from kiwifruit peels is particularly valuable, since it enables the production of a functional biomolecule from an underutilized waste, adding value to kiwi processing waste within a circular biorefinery perspective.

3.1 | Molecular Docking

Molecular docking calculations were performed as a complementary approach to examine possible interaction patterns between

actinidin and the ions composed of the evaluated ILs. The interactions between actinidin and the ions of the cholinium-based ILs evaluated experimentally, namely $[N_{111(2OH)}]^+$, Br^- , Cl^- , $[\text{NTf}_2]^-$, $[\text{DHC}]^-$, $[\text{DHP}]^-$, $[C_1CO_2]^-$, and $[\text{Bit}]^-$, were analyzed. The binding affinity values obtained for the best docking poses are summarized in Figure 2, while the corresponding interaction and binding conformations are presented in Table S1. The absolute docking affinity energies between the ions-IL and actinidin follow the order $[\text{Bit}]^- (-6.1) > [C_1CO_2]^- (-5.4) > [\text{DHP}]^- (-4.4) > [\text{DHC}]^- (-3.4) > [\text{NTf}_2]^- (-3.3) > \text{Cl}^- (-1.1) = \text{Br}^- (-1.1)$, in agreement with the experimental results on the actinidin activity (cf. Figure 1). The calculated docking scores varied markedly among the investigated anions, indicating differences in their predicted interaction propensity toward actinidin. In general, these differences were consistent with the experimental ranking of proteolytic activity, particularly for $[\text{Bit}]^-$ and $[C_1CO_2]^-$, which exhibited the most favorable docking scores and were also among the best-performing extraction systems.

Among the anions investigated, $[\text{Bit}]^-$ and $[C_1CO_2]^-$ exhibited the most favorable affinity values during extraction, followed by $[\text{DHP}]^-$ and $[\text{DHC}]^-$, whereas Br^- and Cl^- showed much weaker interactions. $[\text{NTf}_2]^-$, although presenting a relatively favorable docking value, showed a distinct interaction pattern. These binding values are consistent with the experimental screening, in which $[N_{111(2OH)}][\text{Bit}]$ and $[N_{111(2OH)}][C_1CO_2]$ led to the highest proteolytic activities after extraction, while halide-based ILs were less effective. The agreement between the docking ranking and the experimental screening suggests a correlation between the predicted ion-protein interactions and the observed extraction performance [36, 37]. However, these calculations do not account for solvent organization, ion-water interactions, biomass-protein interactions, or the complex microenvironment of aqueous IL solutions and should therefore be interpreted as supportive molecular-level evidence. Analysis of the interaction profiles further indicated that most ions interacted with actinidin mainly through hydrogen bonding and other non-covalent contacts involving polar amino acid residues. In contrast, the extent and localization of these interactions varied with the ion structure, particularly for the more strongly interacting anions. Thus, the beneficial effect of some ILs may result from a combined contribution of enhanced protein solubilization and more favorable protein-solvent interactions. These solvents may facilitate the actinidin extraction from the kiwi peel matrix. Furthermore, it may provide a hydration environment more conducive to preserving catalytic activity in the resulting extract [38, 39].

Actinidin is a cysteine protease; consequently, interactions near its catalytic site have also been observed. The docking calculations showed that the most promising anions interacted near residues associated with the active site. Such interactions may either preserve the native arrangement of catalytically relevant residues or promote subtle conformational changes that maintain enzymatic function [40]. On the other hand, ions showing poor affinity or less favorable binding distribution may be less effective in stabilizing the enzyme, which is in agreement with their lower experimental performance. The molecular docking results obtained in the present study are consistent with previous reports, showing that solvent effects on enzymatic systems depend strongly on the identity of the interacting ions and the region of the protein surface where

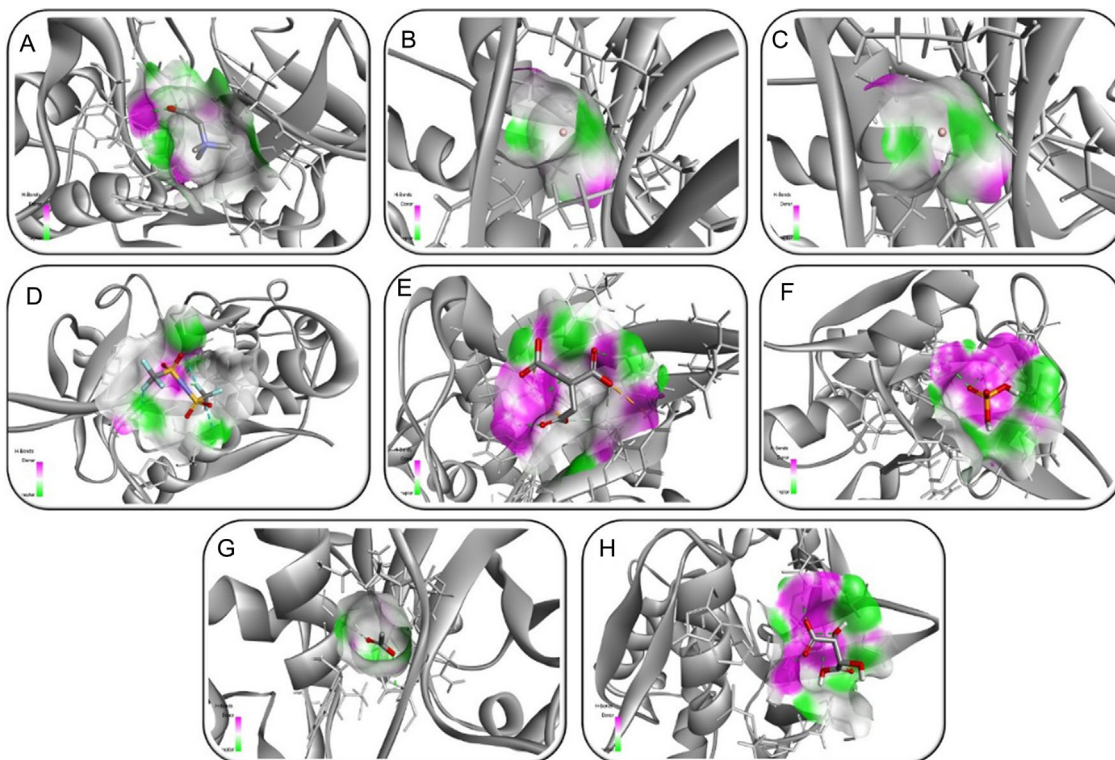


FIGURE 2 | Molecular docking affinity for actinidin with different ionic liquid ions: (A) $[N_{111}(2OH)]^+$, (B) Br^- , (C) Cl^- , (D) $[NTf_2]^-$, (E) $[DHC]^-$, (F) $[DHP]^-$, (G) $[C_1CO_2]^-$, and (H) $[Bit]^-$.

binding occurs. Barbosa et al. [41] for example, showed that different phosphonium-based ILs ions interacted with distinct regions of *Burkholderia cepacia* lipase and that these differences were directly reflected in enzymatic performance, in which the strength of binding, localization, and the nature of the interaction are critical for explaining whether a given ion stabilizes or perturbs enzyme function. Toledo et al. [42] in their study on the activation of laccase in a choline-based deep eutectic solvent (DES), demonstrated that most of the DES components form hydrogen-bonding interactions with the enzyme and that ligands with stronger absolute affinity values were generally associated with better enzymatic performance. Buarque et al. [23] reported a study on the *Yarrowia lipolytica* hexokinase, in which the docking analysis further showed that stronger affinity values can be associated with conformational perturbation or inhibition when the interacting species gains access to functionally sensitive regions of the enzyme.

3.2 | Optimization of Operational Parameters for Actinidin Extraction

After selecting $[N_{111}(2OH)][Bit]$ as the most effective IL for actinidin extraction from kiwifruit peels in the preliminary screening, the influence of extraction time (5 to 90 min) was investigated under fixed conditions of 50 mM IL, a solid-to-liquid ratio of 1:10 w/w, 25°C, and 400 rpm. As shown in Figure 3a, proteolytic activity increased from $0.984 \pm 0.052 \text{ U L}^{-1}$ at 5 min to $1.245 \pm 0.012 \text{ U L}^{-1}$ at 30 min, indicating that the prolongation of contact time initially favors the transfer of actinidin from the kiwi peel matrix to the extraction medium. This observation is compatible with the progressive increase in mass transfer as the solvent remains in contact with the biomass for longer periods. The electrophoretic profiles obtained

under the different extraction times are shown in Figure S1. A similar kinetic trend was reported by Feitosa et al. [13] for protein extraction using aqueous $[N_{111}(2OH)][Bit]$, in which a rapid initial extraction stage was followed by a slower approach to equilibrium. Nevertheless, no substantial difference was observed in proteolytic activity after 30 min, and the values obtained from 30 to 90 min remained the same. At this stage, the driving force for mass transfer weakens, and the extraction rate tends to stabilize as the system approaches equilibrium. This behavior may also indicate that the readily extractable fraction of actinidin had already been depleted, meaning that no significant additional amount remained available for extraction [5]. Therefore, although short extraction times were insufficient to maximize enzyme recovery, extending the extraction time beyond 30 min provided no practical benefit. Thus, 30 min was considered the most appropriate extraction time, as it ensured the highest activity while avoiding unnecessary increases in processing time and operational cost [5].

The influence of stirring speed (100 to 800 rpm) on actinidin extraction was evaluated using 50 mM $[N_{111}(2OH)][Bit]$, a solid-to-liquid ratio of 1:10 w/w, 25°C, and an extraction time of 30 min. As shown in Figure 3b, proteolytic activity increased progressively with agitation speed, rising from approximately $1.219 \pm 0.035 \text{ U L}^{-1}$ at 100 rpm to a maximum of $1.968 \pm 0.036 \text{ U L}^{-1}$ at 600 rpm. The corresponding SDS-PAGE profiles for the different stirring speeds are presented in Figure S1. As shown, increasing agitation enhanced the extraction of the target enzyme from the vegetal matrix. This effect can be attributed to improved solid-liquid contact, reduced external mass-transfer resistance, and increased solvent penetration into the biomass. In such matrices, higher agitation may promote partial disruption or loosening of the biomass, increasing surface

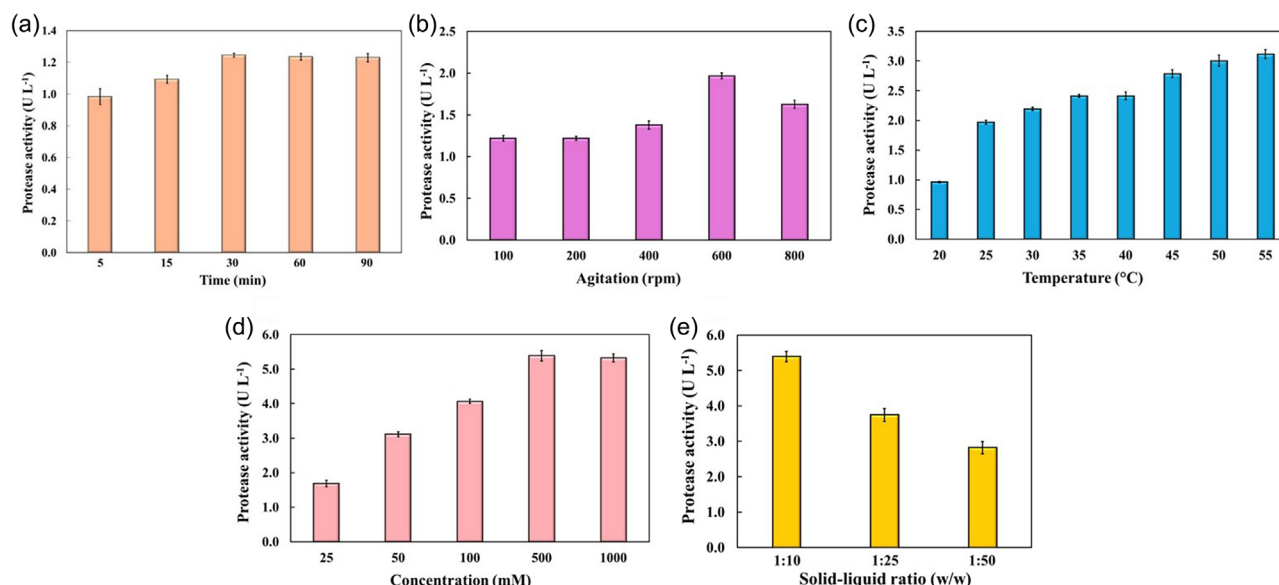


FIGURE 3 | Influence of the main operational parameters on the proteolytic activity (U L⁻¹) of actinidin extracted from kiwifruit (*A. deliciosa*) peel using aqueous [N_{111(2OH)}][Bit]: (a) extraction time, (b) stirring speed, (c) temperature, (d) [N_{111(2OH)}][Bit] concentration, and (e) solid-to-liquid ratio. Extraction conditions were as follows: (a) 50 mM IL, solid-to-liquid ratio of 1:10 (w/w), 400 rpm, and 25°C; (b) 50 mM IL, solid-to-liquid ratio of 1:10 (w/w), 30 min, and 25°C; (c) 50 mM IL, solid-to-liquid ratio of 1:10 (w/w), 600 rpm, and 30 min; (d) solid-to-liquid ratio of 1:10 (w/w), 600 rpm, 45°C, and 30 min; and (e) 500 mM IL, 600 rpm, 45°C, and 30 min.

area and facilitating access to biomolecules that are entrapped or less accessible within the tissue. Consequently, the transfer of soluble protein fractions to the [N_{111(2OH)}][Bit]-containing medium becomes more efficient, as reflected by the higher proteolytic activity values obtained.

The increase observed up to 600 rpm demonstrates that the positive effects of agitation on matrix accessibility and mass transfer outweigh any possible negative effects on enzyme stability. However, when the stirring speed was further increased to 800 rpm, proteolytic activity decreased, indicating that more intense agitation is no longer beneficial. Although stronger agitation can further intensify turbulence and solvent accessibility, it may also expose the extracted enzyme to higher hydrodynamic shear forces and mechanical stress, potentially disrupting its native conformation and reducing catalytic performance [43, 44]. Therefore, the decrease observed at 800 rpm suggests that the system reached a threshold beyond which the gains in protein recovery were offset by losses in enzymatic stability.

The influence of temperature (20 to 55°C) on actinidin extraction was evaluated using 50 mM [N_{111(2OH)}][Bit], at a solid-to-liquid ratio of 1:10 w/w, under stirring at 600 rpm for 30 min (Figure 3c). The SDS-PAGE profiles obtained under the evaluated temperatures are shown in Figure S1. Proteolytic activity increased notably from 20 to 55°C, increasing from approximately 0.96 ± 0.014 U L⁻¹ to a maximum of 3.13 ± 0.076 U L⁻¹. This improvement can be attributed to improved mass transfer, increased molecular diffusion, and the lower viscosity of the IL-containing medium, all of which facilitate solvent penetration into the plant matrix [30, 45, 46]. Moreover, moderate heating may weaken non-covalent interactions between the protein and the biomass components, thereby increasing actinidin solubilization and recovery [47].

A similar temperature-dependent behavior has been reported in other IL-based protein extraction systems. Wang et al. [48] observed that increasing temperature initially improved protein extraction, attributed to reduced IL viscosity and the consequent enhancement of diffusion and solubility in the extraction medium. However, the authors also reported that further increases in temperature were detrimental to the process, likely due to reduced protein stability [48]. It is important to note that higher temperatures were not evaluated, as actinidin is known to be denatured/inactivated at ≥60°C, which could compromise its structural integrity and proteolytic activity [49]. As shown in Figure 3d and Table 2, the effect of [N_{111(2OH)}][Bit] concentration was evaluated on the basis of proteolytic activity, total protein concentration, protein yield, and specific proteolytic activity. Increasing the IL concentration from 25 to 500 mM increased the total protein concentration from 458.7 to 1115.3 mg L⁻¹ and the protein yield from 4.59 to 11.15 mg g⁻¹ biomass, representing a 2.43-fold increase in protein recovery. Over the same concentration range, proteolytic activity increased from 1.685 to 5.388 U L⁻¹, corresponding to a 3.20-fold increase. Consequently, specific proteolytic activity increased from 0.00367 to 0.00483 U mg⁻¹ protein. The effect of IL concentration was evaluated at a solid-to-liquid ratio of 1:10 w/w, 600 rpm, 45°C, and 30 min. The electrophoretic profiles obtained at different [N_{111(2OH)}][Bit] concentrations are presented in Figure S1.

The effect of [N_{111(2OH)}][Bit] concentration was nonlinear across the evaluated range. Increasing the concentration from 25 to 50 mM markedly enhanced total protein recovery, with the protein concentration rising from 458.7 to 895.4 mg L⁻¹. In contrast, specific proteolytic activity decreased slightly from 0.00367 to 0.00348 U mg⁻¹ protein, indicating that the additional protein recovered at 50 mM did not produce a proportional increase in proteolytic activity. Further increases in [N_{111(2OH)}][Bit] concentration

TABLE 2 | Effect of $[N_{111(2OH)}][\text{Bit}]$ concentration on proteolytic activity, total protein concentration, specific proteolytic activity, and protein yield during extraction from kiwifruit (*Actinidia deliciosa*) peel. Extraction conditions: solid-to-liquid ratio of 1:10 (w/w), 600 rpm, 45 °C, and 30 min.

$[N_{111(2OH)}][\text{Bit}]$	Protease activity, U L^{-1}	Protein, mg L^{-1}	Specific activity, U mg^{-1}	Protein yield, mg g biomass^{-1}
25 mM	1.685 ± 0.098	458.7 ± 4.5	0.00367 ± 0.00181	4.59 ± 0.09
50 mM	3.115 ± 0.076	895.4 ± 5.1	0.00348 ± 0.00185	8.95 ± 0.11
100 mM	4.068 ± 0.061	966.1 ± 6.4	0.00421 ± 0.00179	9.66 ± 0.08
500 mM	5.388 ± 0.150	1115.3 ± 8.9	0.00483 ± 0.00186	11.15 ± 0.14
1000 mM	5.323 ± 0.117	1119.2 ± 9.1	0.00476 ± 0.00201	11.19 ± 0.16

resulted in a progressive rise in specific proteolytic activity, reaching $0.00421 \text{ U mg}^{-1}$ protein at 100 mM and a maximum of $0.00483 \text{ U mg}^{-1}$ protein at 500 mM. This concentration-dependent profile reveals distinct effects of the IL on protein recovery and enzymatic functionality, with the highest functional performance observed at 500 mM. Above 500 mM, increasing the $[N_{111(2OH)}][\text{Bit}]$ concentration produced minor changes in protein recovery. Total protein concentration increased from 1115.3 to 1119.2 mg L^{-1} between 500 and 1000 mM, while the protein yield remained essentially constant at 11.15–11.19 mg g^{-1} biomass. Conversely, proteolytic activity decreased slightly from 5.388 to 5.323 U L^{-1} , with a concomitant reduction in specific proteolytic activity from 0.00483 to 0.00476 U mg^{-1} protein. The stabilization of protein recovery at concentrations above 500 mM, accompanied by the absence of further gains in proteolytic activity, defines 500 mM as the concentration at which maximum protein recovery and proteolytic performance were effectively achieved. Feitosa et al. [13] evaluated the extraction of protease inhibitor proteins from Brazilwood seeds, in which IL concentration was identified as a significant process variable, and the best extraction performance was achieved at an intermediate $[N_{111(2OH)}][\text{Bit}]$ concentration (5.42 wt%), rather than at the highest solvent levels evaluated. These results corroborate the interpretation that the effect of $[N_{111(2OH)}][\text{Bit}]$ is not proportional to its concentration and that an optimum solvent range exists in which protein recovery is optimized.

A strong correlation was observed between actinidin recovery and the solid-to-liquid ratio under the optimized extraction conditions (500 mM $[N_{111(2OH)}][\text{Bit}]$, 600 rpm, 45°C, and 30 min), as shown in Figure 3e. The corresponding SDS–PAGE profiles are shown in Figure S1. The highest proteolytic activity after actinidin extraction was obtained at a ratio of 1:10 w/w, reaching $5.388 \pm 0.15 \text{ U L}^{-1}$, whereas further increases in solvent proportion led to progressively lower activity values, decreasing to approximately $3.74 \pm 0.19 \text{ U L}^{-1}$ at 1:25 w/w and $2.84 \pm 0.17 \text{ U L}^{-1}$ at 1:50 w/w. Thus, it can be reported that the proteolytic activity of the extracted actinidin depended on maintaining an adequate balance between the amount of biomass and the extracting medium.

At 1:10 w/w, the amount of $[N_{111(2OH)}][\text{Bit}]$ solution was sufficient to promote efficient wetting of the biomass, good solvent penetration into the matrix, and effective solubilization of actinidin, while still preserving a sufficiently concentrated extract. In addition, excessive solvent proportions may decrease the frequency of effective interactions between the target biomolecule and the extraction medium relative to the amount of biomass

processed, thereby reducing the apparent process efficiency. Ali et al. [5] also demonstrated that actinidin extraction from kiwifruit is strongly influenced by the solid-to-liquid ratio. Using sodium phosphate buffer (0.1 M, pH 7.0), the authors evaluated the ratios 1:0.5, 1:1, 1:1.5, 1:2, and 1:3 (w/v), and reported different activity values. Although the optimal ratio reported in that study differed from that observed here, likely due to differences in the extraction medium and procedure, both results reinforce that actinidin recovery is highly sensitive to phase proportion. It is important to note that the solid-to-liquid ratio of 1:5 w/w was also used; however, no reliable results could be obtained since the system exhibited excessive viscosity. Under this condition, the high solids loading likely hindered homogenization, limited solvent diffusion through the biomass, and impaired the transfer of actinidin from the kiwifruit peel to the $[N_{111(2OH)}][\text{Bit}]$ -containing medium. Moreover, reduced solvent availability may have accelerated local saturation, further restricting protein solubilization.

3.3 | Isolation of Actinidin and IL Recovery

Although the optimized aqueous solution of $[N_{111(2OH)}][\text{Bit}]$ proved effective for the extraction of active actinidin from kiwifruit peels, the practical implementation of this approach requires a downstream strategy able to (i) isolate the protease under mild conditions, (ii) remove the IL from the protein-rich fraction, and (iii) recover both water and IL for reuse [50, 51]. Considering that actinidin is a cysteine protease whose functionality depends on preservation of its native conformation, subsequent processing steps should avoid excessive thermal, chemical, or interfacial stresses. Furthermore, the IL-rich extract can provide a favorable medium for the preservation of actinidin. This possibility is supported by the literature describing cholinium-based ILs as biocompatible media for proteins and enzymes. A recent review by Sindhu et al. [52] described that these solvents can enhance protein solubility, support structural integrity, preserve enzymatic activity, and, in some cases, even contribute to longer-term storage performance, depending on ion combination and operating conditions. Tien et al. [53] demonstrated that cholinium amino acid ILs can improve the thermal and conformational stability of proteinase K, demonstrating that properly selected cholinium-based media can provide a stabilizing micro-environment for enzymes. Likewise, Khachatryan et al. [54] cholinium-based ILs have also been reported to modulate protein stability in a structure-dependent manner, reinforcing the idea that these solvents can act as extraction media and as preservation-compatible environments.

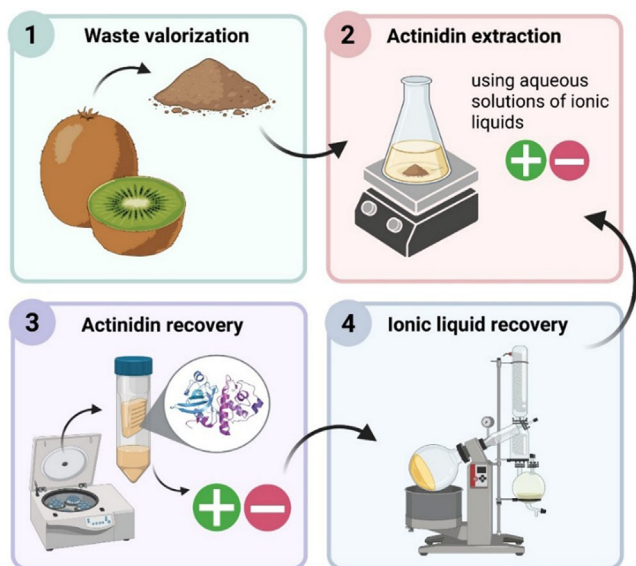


FIGURE 4 | Flowchart of the proposed integrated platform for actinidin recovery from kiwifruit peel: (1) waste valorization, (2) actinidin extraction using aqueous ionic liquid solutions, (3) recovery of the actinidin-rich fraction, and (4) recovery and reuse of the ionic liquid-rich phase.

In this context, a potential recovery pathway can be proposed (Figure 4). Following solid–liquid extraction, the crude extract could be centrifuged to remove suspended biomass particles. The supernatant, containing actinidin, water, $[N_{111}(2OH)][Bit]$, and low-molecular-weight coextracted compounds, could then be subjected to ultrafiltration using a membrane with an appropriate molecular weight cut-off to retain actinidin while allowing the aqueous IL phase and other small solutes to permeate [55]. Since actinidin has a molecular mass of approximately 24 kDa, this approach is expected to yield a protein-enriched retentate and an IL-rich permeate [14].

The IL-rich permeate obtained after ultrafiltration/diafiltration would contain mainly water, $[N_{111}(2OH)][Bit]$, and other low-molecular-weight compounds. From this stream, water could be recovered by evaporation under reduced pressure at a mild temperature, allowing its condensation and reuse, while the non-volatile IL remains in the concentrated phase. The recovered $[N_{111}(2OH)][Bit]$ could then be reconditioned, if necessary, by dilution to the target concentration and analytical control of pH and purity before reusing in a new extraction cycle. This approach would reduce solvent consumption and improve the process's economic and environmental performance. In general, this process scheme provides a feasible basis for integrating extraction, enzyme recovery, and IL recovery into a more sustainable platform for kiwifruit waste valorization [56].

Recent studies from our research group confirm the feasibility of recovering and reusing ILs in integrated extraction platforms. Polesca et al. [57] reported that the supernatant-rich IL and biopolymers extracted from chicken feathers could be recovered by removing volatile compounds under vacuum, allowing the IL to be reused in three successive cycles, with a final mass recovery of 82% for the IL. In another study by Polesca et al. [55] using an aqueous solution of the $[N_{111}(2OH)][C_1CO_2]$, the IL was recovered

and reused over four successive cycles, with an IL recovery yield of 95 wt% and no significant loss in keratin recovery performance. Additionally, the results from Ninomiya et al. [58] and Nakasu et al. [59] confirmed that $[N_{111}(2OH)][C_1CO_2]$ could be reused for at least five cycles while maintaining its chemical structure and process performance. Outeiriño et al. [60] reported the recovery and reuse of cholinium glycinate in the treatment of brewery spent grain, achieving approximately 97% IL recovery and demonstrating effective solvent reuse over five consecutive cycles. To the best of our knowledge, experimental studies specifically addressing the recovery and reuse of $[N_{111}(2OH)][Bit]$ are still scarce. Nevertheless, $[N_{111}(2OH)][Bit]$ -based systems have already been integrated into recovery schemes, such as aqueous biphasic platforms for dye separation, showing that the target compound can be separated from the $[N_{111}(2OH)][Bit]$ -rich phase [61]. These results, although not yet constituting a full reuse study for $[N_{111}(2OH)][Bit]$, demonstrate the technical plausibility of developing recovery and reuse strategies for this solvent. Therefore, a similar approach based on mild protein separation, followed by recovery and reconditioning of the $[N_{111}(2OH)][Bit]$, appears realistic and represents a relevant next step toward the practical implementation of actinidin extraction from kiwifruit waste.

3.4 | Sustainability Assessment

To broaden and validate the development of a more sustainable approach to the proposed process, the actinidin extraction platform was analyzed using the Path2Green framework, a recent sustainability assessment tool specifically designed for biomass extraction processes. Unlike more generic sustainability metrics, Path2Green evaluates extraction routes through 12 green extraction principles that cover the full process chain, from biomass sourcing and transport to solvent selection, purification, energy demand, application, and waste management [62]. Thus, this framework enables a more structured and extraction-oriented sustainability analysis of the proposed process. Within this context, some aspects of the present work are particularly favorable, reaching a final Path2Green score of 0.392, indicating a moderately sustainable extraction route (Figure 5).



FIGURE 5 | Path2Green sustainability score of the actinidin extraction platform and graphical representation of the 12 green extraction principles [62].

The Path2Green assessment highlighted both clear advantages and limitations of the proposed extraction platform, as shown in Table S2. The strongest positive contribution came from the biomass criterion (+1.00), since kiwi peels are an underutilized agro-industrial waste product, and their recovery is fully consistent with the circular use of biomass. Transport also scored positively (+1.00), as the biomass was locally sourced, reducing the environmental burden associated with logistics. The choice of solvent was another key strength (+1.00), given that the extraction was performed using aqueous choline-based ILs instead of conventional hazardous organic solvents [63, 64]. Purification (+1.00) and post-treatment (+1.00) also contributed positively, with the process designed to produce a functional proteolytic extract without requiring extensive downstream purification or additional conditioning steps. The application criterion was positively scored (+0.66), reflecting the recognized relevance of actinidin in food-related processes, particularly in tenderizing meat and coagulating milk; in nutrition and health, in dietary formulations and supplements; in pharmaceuticals, in healing creams and for antimicrobial action; and in biotechnology, in protein modification and hydrolysis [6, 32]. On the other hand, some criteria revealed opportunities for improvement. The pretreatment received a slightly negative score (−0.20) due to the drying and grinding of the biomass before extraction. The process has so far been demonstrated only on a laboratory scale and in batch mode and therefore received the lowest score (−1.00), while the yield (0.00) remained neutral, as the extraction performance was evaluated based on proteolytic activity. Energy (0.00) was also considered neutral, even though the extraction was carried out under relatively mild conditions; the process still includes steps dependent on heating from nonrenewable sources. Repurposing (0.00) and waste management (0.00) also remained neutral, although solvent and water recovery were proposed as viable purification strategies; these were not experimentally validated in this study.

A comparison with the study by Lazarević et al. [65] is also relevant, as these authors reported a Path2Green score of 0.363 for an integrated extraction platform based on cholinium-based IL systems. The slightly higher score achieved in this study is mainly due to the significant contributions of waste biomass recovery and solvent selection, while the lack of experimental solvent recycling and the limited scalability of the process still constrain its overall sustainability. Therefore, the results presented here corroborate the idea that the proposed extraction route already shows promise from a sustainability perspective, while also identifying the key areas for improvement in future developments, namely scaling up the process, validating solvent recovery, and enhancing post-extraction circular strategies.

The extraction platform developed is also particularly aligned with SDG 12 (Responsible Consumption and Production), as it adds value to kiwi peel, an agro-industrial waste product, and promotes more efficient use of biomass and the circular economy. It also aligns with SDG 9 (Industry, Innovation, and Infrastructure) by proposing an innovative extraction method based on biocompatible cholinium-based ILs, and with SDG 13 (Climate Action) by promoting processing strategies that reduce the environmental impact associated with conventional extraction and waste-disposal practices.

4 | Conclusions

This study demonstrated the feasibility of recovering an actinidin-containing proteolytically active protein fraction from kiwifruit (*A. deliciosa*) peels using aqueous solutions of cholinium-based ILs. Among the evaluated systems, $[N_{111}(2OH)][Bit]$ showed the best overall performance. During the initial screening, this IL increased total protein concentration from 176.8 to 384.5 mg L^{−1} and protein yield from 1.77 to 3.85 mg g^{−1} biomass relative to the phosphate-buffer control. Proteolytic activity increased from 0.405 to 1.245 U L^{−1}, while specific proteolytic activity increased from 0.00229 to 0.00324 U mg^{−1} protein, indicating that the improved extraction performance was accompanied by a higher proteolytic activity per unit of recovered protein. Molecular docking provided complementary support for the experimental trends, showing anion-dependent differences in predicted ion–protein interactions. Process optimization identified 500 mM $[N_{111}(2OH)][Bit]$, a solid-to-liquid ratio of 1:10 w/w, 600 rpm, 45°C, and 30 min as the most suitable extraction conditions. Under these conditions, proteolytic activity reached 5.388 U L^{−1}, with a total protein concentration of 1115.3 mg L^{−1}, a protein yield of 11.15 mg g^{−1} biomass, and a specific proteolytic activity of 0.00483 U mg^{−1} protein. SDS–PAGE revealed a protein band consistent with the molecular mass of actinidin, while the presence of additional bands confirmed the coextraction of other proteins.

Beyond the experimental findings, this work highlights the significant potential of kiwifruit peel as an underutilized source of high-value enzymes. Moreover, the Path2Green score of 0.392 indicates that the proposed platform already represents a moderately sustainable extraction route, while also highlighting scale-up, energy optimization, and solvent-recovery validation as relevant targets for future improvement. Future work should prioritize integrated downstream processing for enzyme isolation and IL reuse, optimization of pretreatment, energy demand, scalability, and circular postextraction strategies, as highlighted by the Path2Green assessment. In parallel, technoeconomic, life cycle, and broader sustainability analyses will be crucial to confirm the practical viability of the process and guide its progression toward industrial implementation.

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that support the findings of this study are available in the material (Supporting Information) of this article.

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Supporting Information

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