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"Innovating Multi-Functional Protein & Peptide Solutions: Sustainable, Scalable, and High-Performance Ingredients for Diverse Applications"

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1. Introduction

Proteins and protein hydrolysates have been utilised in the global cosmetics industry to enhance formulation efficacy and performance for many years. One such protein is keratin that, once hydrolysed, has been used extensively in haircare formulations to improve conditioning, hair fibre strength, and shine amongst other cosmetic outcomes. This fibrous structural protein has historically been extracted and chemically or enzymatically hydrolysed from several animal or even human (hair) sources. However, after 2005, the use of human origin materials was banned in cosmetic products sold to the EU market, leaving only animal materials (wool, feathers, hooves, and horn) as viable sources of keratin and keratin hydrolysates (1). However, with evolving consumer demands for cleaner and more sustainable products, animal sourced ingredients have significantly diminished in popularity, creating a keratin source issue for the industry. Plant-derived protein hydrolysates that mimic keratin have been incorporated into various high-volume haircare products and have therefore somewhat addressed this issue. However, they do not fully deliver in terms of performance and have been implicated in some incidences of allergenicity leaving the market searching for further alternatives (2,3). Similarly, synthetic keratin-derived peptides manufactured using techniques such as solid-phase peptide synthesis (SPPS), only partially address the issue too (3). Whilst this methodology offers customisable and scalable production of small peptides, these peptides have a high price per kilo, limiting their use to premium products only. Further, SPPS becomes cost prohibitive when larger peptides are of interest and so drastically narrows the scope of what can be viably made and sold within the cosmetics industry. More relevant to evolving consumer demands however, the solvents and reagents involved in SPPS are environmentally damaging and unsustainable, resulting in recent EU bans for some critical solvents.

The remaining solution to the keratin source issue lies with biotechnology, harnessing scalable microbial fermentation to sustainably produce animal free keratin, hydrolysates, and defined/customised peptides. However, traditional biotechnology approaches are very expensive and typically take years to realise, owing to long development cycles associated with necessary microbial strain engineering to achieve acceptable yields. This problem is made worse

when ‘challenging’ proteins are of interest; keratin being one such protein, due to its highly repetitive primary structure and overall hydrophobicity (1).

Given these critical flaws, biotechnology would not appear to be a suitable source. However, we have developed a rationally designed modular platform molecule approach (Figure 1) to unlock biotechnology for the cosmetics industry. This approach removes the need for microbial strain engineering completely, as we demonstrate high purified yields (12 g/L) of a keratin-based protein (KBP) obtained from a standard commercially available *E. coli* expression strain. Further, the platform facilitates the custom design of multifunctional proteins, defined peptides and peptide blends in a highly water-soluble format. In combination, development times of new or challenging proteins of interest, such as keratin, are reduced from years to months and development costs are reduced significantly.

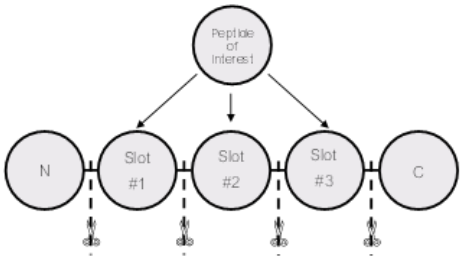


Figure 1. Representative schematic of the platform molecule. The platform molecule contains multiple regions, referred to as slots, that allow integration of different protein/peptide motifs. In this study human keratin peptides were integrated. Intrinsic platform components increase stability during expression and purification. Proteins designed within the platform can accommodate specific digestion, releasing single or multiple peptides of interest.

2. Materials and Methods

Recombinant KBP Expression

A plasmid encoding KBP was cloned into BL21 (DE3) *E. coli.* To confirm expression of KBP*,* cells were first cultured in 1 L shake flasks in appropriate media supplemented with antibiotics for selection. Protein expression was induced using IPTG when the desired culture density was obtained. With the confirmed overexpression of KBP, cultures were scaled up and incubated in a 5 L fermenter using identical media conditions. After the fermentation ran to completion, the wet cell paste (WCP) was harvested using centrifugation, and the WCP was lysed using mechanical disruption. The resultant lysate was clarified using a further centrifugation step, and the supernatant was retained. KBP was purified from the retained supernatant using column chromatography and tangential flow filtration.

KBP Characteristics

KBP's qualitative characteristics, such as colour, were assessed visually. Additionally, pH and thermostability over time were assessed by exposing KBP to a range of different pH values (3 – 7.5) offered by different buffering systems and controlled temperatures (-20 to +95 °C).

Film Formation

The film-forming properties of purified KBP protein (at 3% w/v) were assessed in minimal formulation with plasticisers (glycerol, glycerol + oleic acid, or glycerol + palmitic acid; all 10% w/v). The KBP plasticiser mixture was poured into moulds, which were allowed to dry and form

films at varying temperatures*. The resultant film thickness was measured using a digital film thickness gauge. In addition, the water and oil permeability of the films was assessed
*Note: Temperature required for forming films depends on plasticiser and water/permeability requirements.

Hydrolysate Formation
KBP (at 10% w/v) was hydrolysed into keratin peptides either enzymatically (papain) or chemically (hydroxylamine). Time-course experiments were conducted to determine peptide release, and peptide accumulation over time was quantified using Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE).

Keratin Peptide Microarray
The 96 well microarray assay was fabricated by conjugating the target keratin and keratin associated protein (KAP) peptides to the well bottom surface. The selective binding of KBP with keratin/KAP target peptides was tested by pipetting fluorophore-tagged (5(6)-Carboxytetramethylrhodamine N-succinimidyl) KBP into the 96-well plate and incubating the proteins for 2 hours at 37 °C. After removing any unbound KBP with washing steps, the relative fluorescence of the microarray plates was measured using a fluorescence reader.

3. Results

3.1. KBP shows solubility across a wide pH of buffers and thermostability across high temperatures without the addition of preservatives.

The one-step IMAC purification of KBP resulted in 95% pure protein that was odourless, colourless and clear. After concentration, the protein exhibited a slightly off-white colour, but it remained clear despite being concentrated 10-fold. Colourless or white-coloured ingredients are often considered ‘purer’ than yellow-coloured ingredients. The lack of colour in liquids remains important in cosmetic formulations, particularly in Asian cosmetics, highlighting the applicability of KBP in these markets (4,5). To assess the ideal range of buffers to use for formulation and testing of the KBP, common buffers were prepared, and the pH was adjusted to a range of 3 to 7.5. KBP was soluble at up to 150 mg/mL in 20 mM HEPES, pH 7.5. Additionally, solubility testing across citric acid-Na_2HPO_4 and HEPES buffers was conducted for use in formulations. It was observed that KBP precipitated when resuspended in the buffer of pH 4 or less, however it was completely soluble within the pH 5-7.5 range which is a commonly used pH range for haircare products (6) and is more viable for skin pH conditions (Table 1).

Table 1. KBP solubility using buffer across a pH range.

Buffer	pH	Protein Solubility
Citric Acid Buffer	3.0	-
Citric Acid Buffer	3.5	-
Citric Acid Buffer	4.0	+
HEPES Buffer	5.0	++
HEPES Buffer	5.5	+++
HEPES Buffer	6.0	+++
HEPES Buffer	7.0	+++
PBS Buffer	5.0	++
PBS Buffer	5.5	+++
PBS Buffer	6.0	+++
PBS Buffer	7.0	+++
PBS Buffer	7.5	+++

Additionally, the thermostability of KBP was investigated by exposing the preservative-free protein to a range of temperatures over time (Table 2). SDS-PAGE analysis of KBP samples did not show signs of degradation throughout 3 freeze-thaw cycles (-20ºC to + 25ºC) when stored in 20 mM HEPES, pH 7.5. Further, KBP tolerated 95°C for 30 minutes showcasing excellent stability. Thermostability for haircare products is vital, as heat is often applied to hair in various forms: hot showers, hair straightening/curling, blow-drying, and products that can withstand this heat allow better application and durability as seen for KBP.

Table 2. KBP thermostability times across temperature ranges.

Temperature (ºC)	Time
+95	30 minutes
+20 to +25	6 months
+4	6 months
-20	1 year

3.2. KBP forms water and oil-resistant films.

Film-forming properties are relevant in cosmetics, especially as an ingredient to form a protective layer over skin, hair and nails. Additionally, films are used for masks, whether hair, face or body, to allow ingredients to interact with the surfaces for an extended period of time, increasing effectiveness. Thus, we tested the film-forming characteristics for KBP to determine its applications within the cosmetics and personal care industries. A volume of 2 mL of KBP (3% w/v) resuspended in HEPES buffer was formulated neat (no additions), with glycerol (10%(w/v)), or a combination of glycerol and oleic acid or palmitic acid (10% (w/v) for both) to determine its resistance to water and oil. All KBP films were oil-resistant, suggesting better maintenance on hair, skin or nails as natural body and scalp oils will not break down the protein. KBP formed water-resistant films when formulated with palmitic and oleic acid, demonstrated by the lack of disruption of the film after water penetration tests which included exposing the film to water for at least 5 minutes then using a cotton wipe to rub the film until it is dry. The neat KBP films were water-permeable, shiny, clear and colourless. The shine, clarity and lack of colour was mirrored in the water-resistant KBP films formulated with oleic acid, making KBP an extremely versatile ingredient in haircare products required to last in humid conditions and

during rinsing, again increasing efficacy of the product. The characteristics shown by KBP films are not only relevant to haircare, but potentially make-up applications where water resistance is a key performance enhancer for products such as mascara, lip stains and even sunscreen, adding value and scope to an already multi-functional ingredient like KBP.

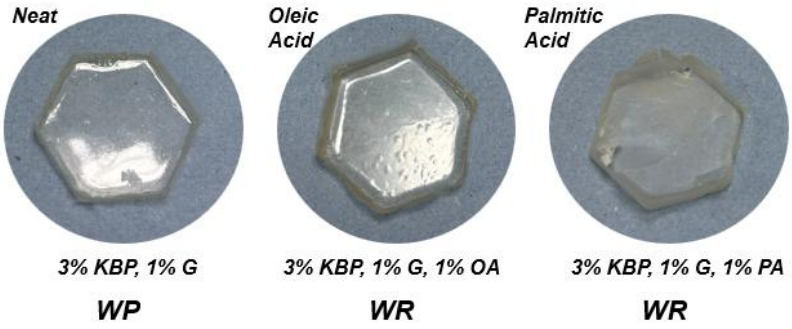


Figure 2. Films formed with KBP. KBP 3% (w/v) was formulated with glycerol, glycerol + oleic acid or glycerol + palmitic acid to create films with different qualitative and functional characteristics. Film thickness was measured as 0.152 mm, 0.150 mm and 0.164 mm for neat, oleic acid and palmitic acid films, respectively.

Table 3. KBP film formation water and oil permeability and qualitative observations.

	Water Permeability	**Oil Permeability**	**Qualitative Observations**
KBP neat	Yes	No	Clear, colourless, shiny, flexible
KBP + oleic acid	No	No	Clear, colourless, shiny, flexible
KBP + palmitic acid	No	No	Cloudy, shiny, more rigid

3.3. Enzymatic and chemical hydrolysis of KBP releases distinctly-sized peptides.

KBP was hydrolysed using enzymatic or chemical methods (papain and hydroxylamine respectively). Papain indiscriminately digested KBP into peptides ranging from ≈ 2 kDa – 25 kDa. Conversely, hydroxylamine hydrolysis showed targeted cleavage, releasing defined peptides from the platform molecule integrating human keratin peptides (Figure 3) thus offering more formulation options for the protein. Prediction of the released peptides through hydroxylamine cleavage was successful, as the peptides observed by SDS-PAGE (Figure 3) aligned with our molecular weight predictions. Predicting protein-to-peptide breakdown underlines that we can control the release of specific peptides in a customised blend presenting alternative manufacturing process options to SPPS. The modular fashion of our platform technology utilised for KBP production can thus be used for larger protein production, not possible due to high costs via SPPS as well as smaller peptides which are directly associated with better hair fibre and skin penetration which is key to producing high end performance products (1).

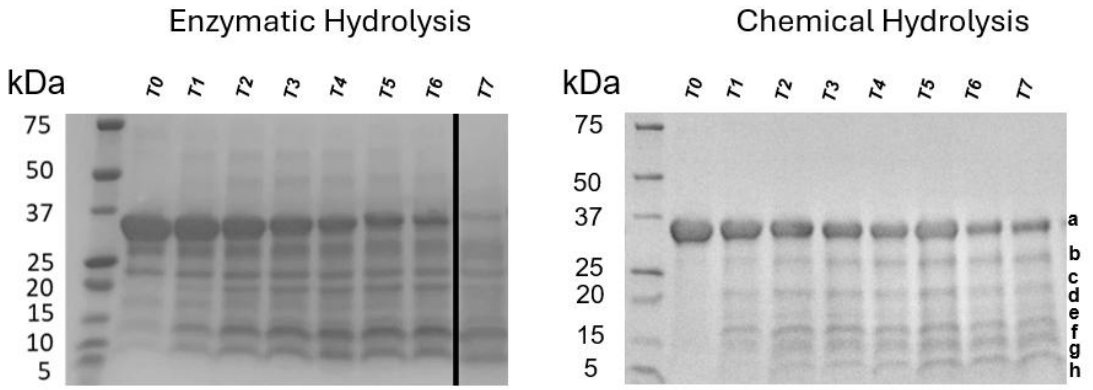


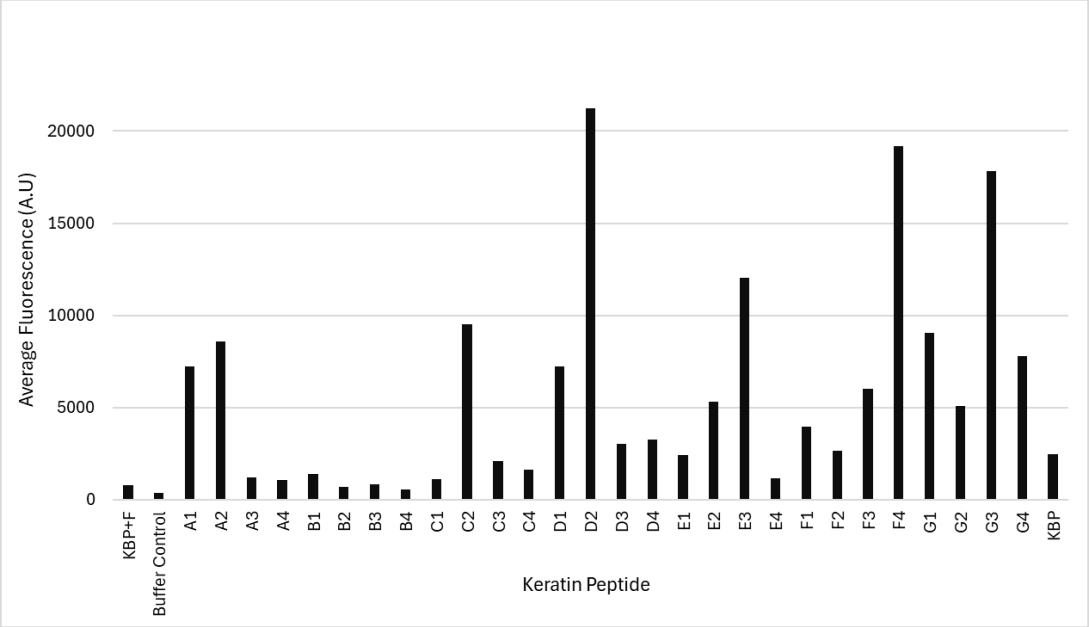
Figure 3. KBP was hydrolysed using papain (enzymatically) or hydroxylamine (chemically) to release peptides with distinct molecular weights over sequential time points. SDS-PAGE analysis of hydrolysed KBP shows protein being broken down peptides ranging from ≈ 2 kDa – 25 kDa. Whilst enzymatic hydrolysis shows random cleavage of KBP into peptides, chemical cleavage shows targeted cleavage of KBP into predicted peptide sizes: a=34.33 kDa, b=28.14 kDa, c=20.26 kDa, d=17.62 kDa, e=16.71 kDa, f=14.07 kDa, g=5.28 kDa, h=2.64 kDa.

3.4. Keratin peptide microarray shows robust selective binding to protein targets found in hair fibres.

The purpose of including proteins and hydrolysates in cosmetic formulation is to enhance the efficacy of the product. Therefore, KBP's potential efficacy as a haircare ingredient was investigated through a microarray method; designed to detect selective binding of KBP (whole and hydrolysed) to a suite of protein targets found in the hair fibre (acidic keratins, basic keratins and keratin-associated proteins). Peptides were derived from the targets and immobilised within the wells of a 96-well plate and relative fluorescence was measured to show binding of the KBP to the immobilised keratin peptides. The obtained results highlight the strong binding of the hydrolysed KBP to basic keratin peptides and keratin associated peptides (D2, E3, F4, and G3) compared to the control wells (Figure 3A). Especially in its hydrolysed form, KBP has strong potential to penetrate the hair fibre and bind to proteins within the hair. This would work to deliver performance outcomes such as increased hair strength and condition. The assay demonstrated selective binding of KBP to common keratin peptides found in hair, with affinity towards D2, E3, F4, and G3. The type of bond was determined to be a strong, disulfide bond, determined through bond disruption after dithiothreitol (DTT) addition (Figure 3B). To determine this, we added a reducing agent, DTT to the 96-well plate. The wells were washed 3x with 50 mM DTT and the results highlight a decrease in binding, particularly between KBP and keratin peptides D2, E3, F4 and G3. The general reduction in relative fluorescence showed that adding DTT broke disulfide bridges, reducing fluorescence as peptides are washed off, which mimics natural keratin-keratin hair bonding, making our results biologically relevant. The highest reduction was observed for the peptides that bound most strongly to our KBP. Figure 3A and 3B also show that there was not a complete reduction in binding as not all the bound KBP was washed off, suggesting that other binding interactions governed by hydrophobic Van der Waals forces, hydrogen or ionic bonds could be present. These results suggest multimodal binding mechanisms of hydrolysed KBP to the targets, directly linked with increased

product retention on human hair. Additionally, the microarray highlights options of formulating with KBP or the hydrolysed form, suggesting the former could coat hair and aid frizzy hair during humid conditions and the latter could penetrate human hair, adding strength.

A



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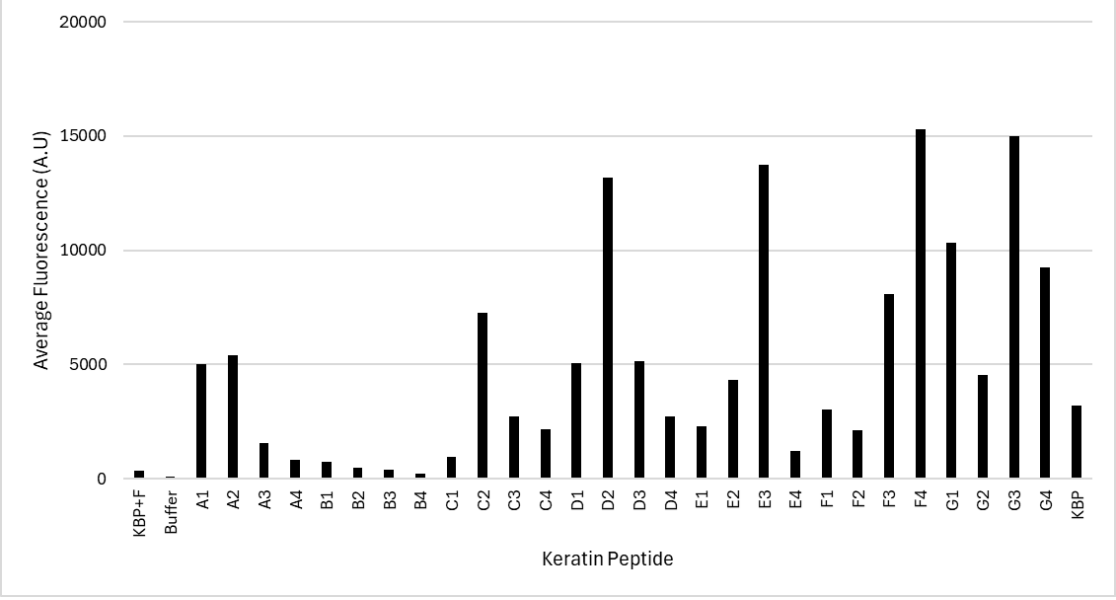

Figure 4. Hydrolysed KBP binding to immobilised keratin peptides shows strong binding to four peptide sequences. The wells of a 96-well plate were coated in keratin and KAP peptide sequences and binding was detected using fluorescence spectroscopy. Control wells did not contain immobilised keratin peptides and included either the KBP plus fluorophore (F), HEPES buffer control, or KBP on its own. **A)** Microarray assay was performed using hydrolysed KBP, and the strongest binding of KBP was found to be to keratin peptides D2, E3, F4, and G3. **B)** Microarray assay was performed, followed by 3x 50 mm DTT washes to remove any disulfide bond KBP from keratin peptides. Where peptides A1-G4 = acidic keratins, basic keratins or keratin-associated peptides and F = fluorophore.

4. Discussion

The results in this paper show viable use of biotechnology to produce high efficacy keratin for the cosmetics industry. Keratin production has always been challenging, due to its sequence complexity and due to animal materials being the primary source of the protein, leaving a gap in the market and developing a need for high-performance, customised keratin peptides (4). Through our platform technology, we highlight a method which breaks the barriers faced in using traditional biotechnology to produce proteins and hydrolysates, such as timescales leading to high costs. The results in this paper emphasise the construction, recombinant expression and purification of KBP. KBP was produced using a plasmid-based platform technology following a one-step affinity purification method to yield >12 g/L of >95% pure protein. This demonstrates the platform's industrial feasibility as high yields like this are uncommon in a standard procedure using commercially available BL21 (DE3) cells (7). Functional characteristics showed the solubility and stability of the KBP at high concentrations of up to 150 mg/mL across cosmetically relevant pH ranges (pH 5–7.5) (6). These characteristics along with its shelf stability across a range of temperatures made it viable for use in long-term product development without loss of functionality. Along with its stability, KBP forms films. These clear, flexible films demonstrated functionality by being water-and-oil-resistant, a characteristic often sought after in formulations to convey barrier protection and even anti-frizz effects. The variation in properties such as the film's rigidity when formulated with palmitic acid and its flexibility and clarity when formulated with oleic acid shows a vital appeal for cosmetics and personal care: personalisation. Depending on formulation, KBP films can be tailored to achieve desired characteristics particularly within the haircare industry.

We demonstrate the possibility of producing proteins and releasing predicted peptides from the platform (Figure 3) with similar efficacy to animal-derived products, however which are completely ethical and animal-free, opening new market opportunities, previously limited due to ethical and religious reasons. Compared to SPPS, where larger peptide production leads to unfeasibly high costs, the scope of our platform is industrially relevant. Additionally, the modular nature of expression allows the integration of multiple keratin motifs into one molecule, meaning the protein is stable throughout and after production and can be comfortably hydrolysed, adding functionality by binding to acidic, basic and keratin-associated peptides demonstrated through a microarray (Figure 4). The relevance of our platform lies in its versatility as our innovation could be applied to motifs beyond keratin. This opens the application for a new selection of ingredients from sustainable sources in the personal care and cosmetics industry, meaning the industry can keep up with the evolving consumer demands.

5. Conclusion

In summary, our platform-based production method offers a sustainable, scalable, and high-yield solution for generating keratin-based proteins (KBP) suitable for cosmetic applications. The platform technology addresses long-standing limitations in recombinant keratin synthesis, such as sequence complexity, by enabling modular protein design and efficient peptide release. As such, this approach opens up a cosmetic toolbox and lays the foundation for next-

generation bioactive ingredients that comply with both regulatory and environmental demands in the personal care industry.

6. References

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