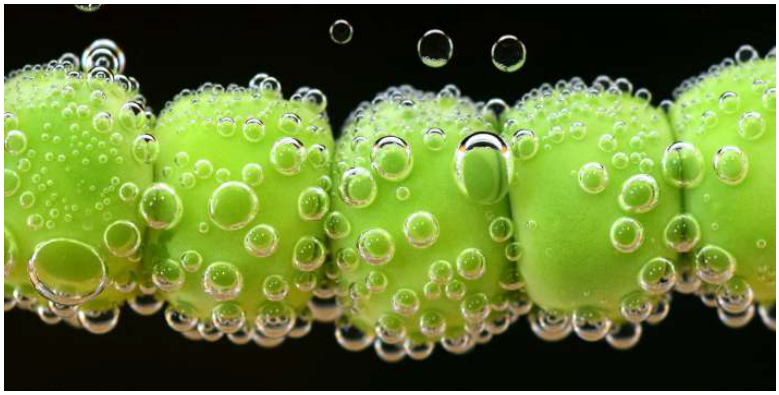
ACB Pisum Sativum Peptide



BACKGROUND

Hydrolyzed proteins such as soy, wheat or oat have been used to impart conditioning benefits and film-forming properties to the hair for decades. These hydrolysates are comprised of random amino acid sequences that aid in improving the elasticity, texture, and hydration of the hair. Recent efforts within the Nutritional Industry have focused on the selection of more precise protein fragments to improve the benefits of supplements. In the course of this research, it has become clear that protein fragments from different sources have varied benefits. To use the verbiage of Malcolm Gladwell, one of the "Outliers" is *Pisum sativum*.

Recently, *Pisum sativum* proteins have attracted the interest of nutrition and health advocates as a plant-based, hypoallergenic protein that yields a high Biological Value (BV). Biological Value is an accurate indicator of the available nutritional potential of a protein. On average, *Pisum sativum* Protein has a 65.4% BV, in comparison to soy protein which only has a 50.0% BV average and wheat protein with only a 49.0% BV average. While it may have had a modest beginning, the enhanced bioavailability of *Pisum sativum* proteins has caught the market by storm as the quality alternative to other vegetable proteins; providing benefits such as high solubility (for easy digestion), enhanced kidney function, and lowering of the blood pressure.

SCIENCE

Proteins are traditionally hydrolyzed using acids, alkalis, and enzymes or some combination there of to produce random amino acid sequences. While traditional methods of hydrolysis are well accepted and effective, they are simplistic efforts to duplicate normal cellular protein catabolism whereby cells digest proteins into specific sequences to meet their nutritional needs. Active Concepts has harnessed the digestive abilities of a proprietary non-GMO bacterial strain, *Lactobacillus bulgaricus,* to produce *Pisum sativum* peptides with a controlled molecular weight of approximately 750 Da.

Pisum sativum protein is a complete source of Essential Amino Acids (EFAs). In fact, *Pisum sativum* has the most balanced amino acid profile of any vegetable protein, consisting of 22 amino acids, notably, rich in lysine[2]. Lysine functions as a vital building block in human biology. Since lysine synthesis does not occur in the body naturally it must be obtained from outside sources, such as protein derived from *Pisum sativum*.

Code Number: 16810

INCI Name: Pisum Sativum (Pea) Peptide
INCI Status: Conforms
REACH Status: Complies
CAS Number: 90082-41-0
EINECS Number: 290-130-6
Origin: Botanical
Processing:
- GMO Free
- No Ethoxylation
- No Irradiation
- No Sulphonation

Additives:
- Natural Antimicrobial: Leuconostoc/ Radish Root Ferment Filtrate
- Preservatives: None
- Antioxidants: None
- Other additives: None

Solvents Used: Water
Appearance: Clear to Slightly Hazy Liquid
Soluble/ Miscible: Water Soluble
Ecological Information:
- 87.30% Biodegradability

Microbial Count:
- < 100 CFU/g, No Pathogens

Suggested Use Levels: 1.0 – 5.0%
Suggested Applications: Hair & Skin Care, Anti-aging, Antioxidant, Hydrating, Smoothing, Volumizing

ACB Pisum Sativum Peptide

BENEFITS

Anti-Aging is the latest trend in Hair Care. ACB Pisum Sativum Peptide provides a potent and cost effective solution by delivering volume and antioxidant protection offsetting the symptoms of hair aging. ACB Pisum Sativum Peptide's film-forming properties render it an effective material for hydrating the hair for a silky feel. Recent demand for anti-aging hair products has prompted formulators to seek out materials and manufacturing methods that will allow targeted claims. ACB Pisum Sativum Peptide reduces the damage caused by free radicals to promote the scalp and follicle health essential producing youthful, voluminous looking hair.

EFFICACY

A series of *in-vivo* and *ex-vivo* studies were performed on volunteers and human hair tresses to evaluate the ability of ACB Pisum Sativum Peptide to provide perceivable benefits to the hair.

The first study study was conducted at Gaston College Technology Center (USA) where the diameter of colored hair was measured at different intervals to determine an increase in hair thickness. Using 60 strands of hair, a 2.0% solution of ACB Pisum Sativum Peptide was applied to each strand. A solution of 2.0% Wheat Hydrolysate in water was used as a positive control for comparison. Immediate results showed an average increase in hair diameter of 14.08% when using the ACB Pisum Sativum Peptide. Four hours after application an average increase of 13.65% was measured when compering the ACB Pisum Sativum Peptide to the control. These results indicate that 2.0% ACB Pisum Sativum Peptide provides thickening benefits to the hair.

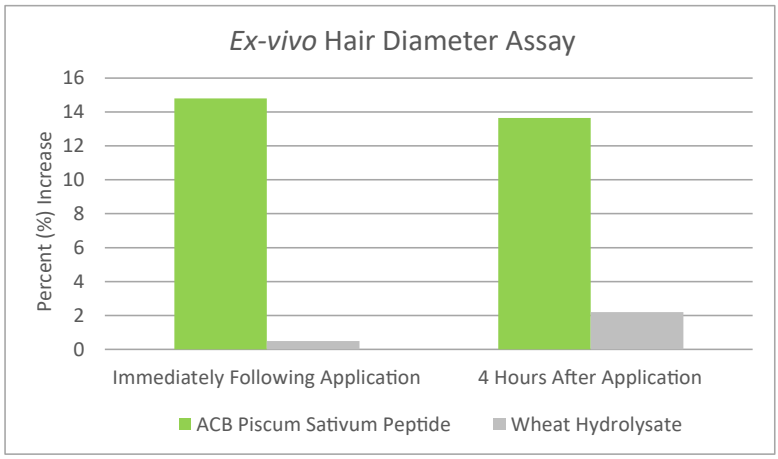


Figure 1. Increase in hair diameter after application of 2.0% ACB Pisum Sativum Peptide in solution compared to the contol of 2.0% Wheat Hydrolysate in solution

Microscopy Imaging of the individual strands were then taken to visually demonstrate the increase in hair diameter achieved when using 2.0% ACB Pisum Sativum Peptide in comparison to 2.0% Wheat Hydrolysate. From these images it can be seen that the ACB Pisum Sativum Peptide is instantly substantive to the hair producing an even film, whereas the Wheat Hydrolysate beads onto the strand. These images further demonstrate the increase in hair diameter achieved when using ACB Pisum Sativum Peptide compared to the Wheat Hydrolysate. ACB Pisum Sativum Peptide is able to effectively thicken the strands for fuller and younger looking hair giving a revolutionary step for anti-aging hair care products.

ACB Pisum Sativum Peptide

Figure 2. Individual strand following immediate treatment with 2.0% Wheat Hydrolysate

Figure 3. Individual strand following immediately treatment with 2.0% ACB Pisum Sativum Peptide

Figure 4. Individual strand four hours after treatment with 2.0% Wheat Hydrolysate

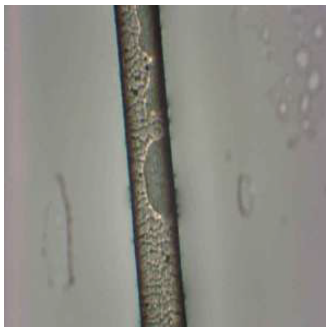


Figure 5. Individual strand four hours after treatment with 2.0% ACB Pisum Sativum Peptide

Increased hydration of the hair is a key benefit of hydrolyzed proteins. As evidenced in an *in-vivo* study, ten (M/F) subjects between the ages of 24 and 37 were instructed to apply either an untreated control, a solution containing 5.0% ACB Pisum Sativum Peptide, or a 5.0% solution containing Wheat Hydrolysate to their hair as a leave-in conditioner, once a day for a week. A DPM 9003 Nova Impedence Meter was used to test the moisture levels on the hair. The results demonstrated a comparable increase in hair hydration on subjects using both a 5.0% solution of ACB Pisum Sativum Peptide and a 5.0% solution of Wheat Hydrolysate.

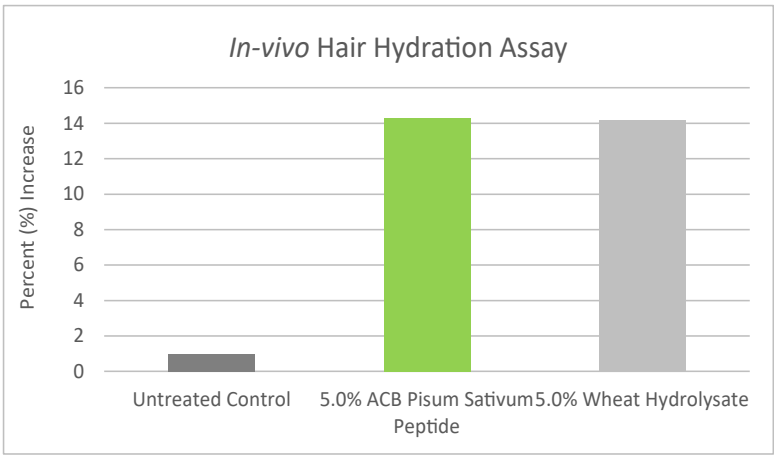


Figure 6. Increase in hair hydration when treated with 5.0% of ACB Pisum Sativum Peptide in solution compared to the contol of 5.0% Wheat Hydrolysate in Solution

ACB Pisum Sativum Peptide

An *in-vivo* half head study was conducted using five participants with a variety of hair types to determine the comparison of using a shampoo and conditioner incorporating 2.0% ACB Pisum Sativum Peptide vs. a control shampoo and conditioner. Each volunteer's hair was photographed before and after washing and blow dry styling with the test and control products. The images of the half head study were used in conjunction with a sensory assessment subjectively rating shine, volume, dry and wet combability, thickness, smoothness, hydration, softness and manageability.

Figure 7. Half-head study to compare hair washed and styled after using a base shampoo and conditioner (left) vs hair washed and styled using a base shampoo and conditioner plus 2.0% ACB Pisum Sativum Peptide (right)

Figure 7 shows that the hair treated with 2.0% ACB Pisum Sativum Peptide appears more voluminous, shiny, soft and healthy than when using the base shampoo and conditioner on their own. Consequently, these results highlight that ACB Pisum Sativum Peptide is capable of enhancing the volume and overall health of the hair perfect for use in anti-aging hair care product lines.

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Materials & Methods

Testing was performed on sixteen total virgin hair swatches. A testing group consisted of four swatches, each of a different ethnicity: European, Afro-American, Asian, and Indian. Four testing groups were observed: untreated, shampoo/conditioner, saturation with rinse, and saturation without rinse. For the shampoo/conditioner testing group, the hair swatches were washed with both a generic shampoo and conditioner formulation incorporating 2.0% **ACB Pisum Sativum Peptide**. For the saturation with rinse testing group, the hair swatches were fully saturated in a 2.0% **ACB Pisum Sativum Peptide** in water solution, allowed to completely air dry, rinsed with 500 grams of deionized water, and allowed to air dry again. For the saturation without rinse testing group, the hair swatches were fully saturated in a 2.0% **ACB Pisum Sativum Peptide** in water solution and allowed to completely air dry.

Testing was performed in a custom 22”x15”x6” smoke chamber. A standard filter pump was placed in the side of the chamber as air supply and standard ceramic filter funnel was placed in the top of the chamber for continuous exposure. Each testing group of hair was hung together in designated 3x3 inch section at the top of the smoke chamber. For stagnant exposure testing, four standard 2 ounce glass retain jars were placed inside the chamber.

One hundred cigarettes were placed into the ceramic filter funnel, with the tip of the cigarettes facing outward. Cigarettes were Riverside brand and all of the cigarette filters were torn off at the base. The cigarettes stood upright tightly packed in the filter funnel and the tips were torched. The hair samples were in the smoke chamber for a total of thirty minutes. For the first ten minutes, continuous air was pulled through the filter pump system and the cigarettes were re-torched when needed. The final twenty minutes, the hair samples underwent stagnant exposure. During stagnant exposure, five cigarettes were placed standing in each of the four retain jars and positioned at each corner of the smoke chamber and the tips of the cigarettes were lit every five minutes. During stagnant exposure, the smoke chamber was fully sealed with the exception of small opening in the top where the original filter funnel was to allow oxygen into the system with minimal smoke loss. After the hair samples completed the time in the smoke chamber, each individual hair sample was placed in a sealed plastic bag for testing.

Hair lipid peroxidation was assessed with the Abcam Lipid Peroxidation (MDA) Assay Kit (ab118970). Samples were taken from the middle portion of the hair strand, totaling 0.2g of tissue weight. To prepare the hair for testing, each hair sample was washed with cold phosphate buffer solution (PBS) and placed in 15ml conical tubes with the MDA lysis buffer. The pestle portion of a dounce grinder and vortex were used to agitate the hair and release the lipids.

The Lipid Peroxidation (MDA) Assay Kit (Colorimetric/Fluorometric) (ab118970) is a convenient tool for sensitive detection of the MDA present in a sample. The MDA in the sample reacts with thiobarbituric acid (TBA) to generate a MDA-TBA adduct. The MDA-TBA adduct can be easily quantified colorimetrically using optical density.

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Results

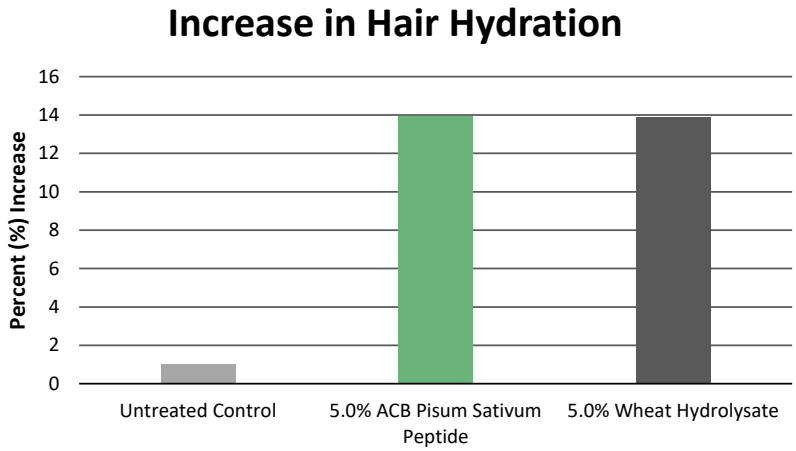

Figure 1. Average Percent Increase in Hydration.

Discussion

Both the innovative hydrolysis method used in the production of **ACB Pisum Sativum Peptide** and the traditional method of hydrolysis induced when creating wheat hydrolysate yield a protein hydrolysate capable of producing hair hydrating benefits at virtually identical levels. This demonstrates a novel approach in hydrolysis, such as the one used in the manufacturing of **ACB Pisum Sativum Peptide** provides just as much moisture as hydrolyzed wheat protein with proven hydration benefits.

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Materials

A. Equipment:	Synergy H1 Microplate reader (BioTek Instuments, Winooski, VT); Gen5 software (BioTek Instuments, Winooski, VT); Pipettes
B. Buffers:	75mM Potassium Phosphate (pH 7.4); Deionized H_2O
C. Reagents:	2,2′-Azobis(2-methylpropionamidine) dihydrochloride (AAPH) (153mM); 6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox®); Fluorescein Sodium Salt (4nM)
D. Preparation:	Pre-heat (37°C) Synergy H1 Microplate reader; Prepare Trolox® standards, sample dilutions, fluorescein solution, and AAPH.
E. Microtitre Plates:	Corning 96 Well Black Side/Clear Bottom Microplates

Methods

Solutions of **ACB Pisum Sativum Peptide** and Trolox® (positive control) were prepared in 75mM potassium phosphate buffer. Materials were prepared at three different concentrations/dilutions. Trolox® was used as a reference for antioxidant capacity and prepared at a concentrations ranging from 12.5µM to 200µM in 75mM potassium phosphate buffer.

For the ORAC assay, 25µL of test material and Trolox® were combined with 150µL of fluorescein in 75mM potassium phosphate buffer and incubated in the Synergy HT Microplate reader at 37˚C for 30 minutes. At the end of the incubation period, 25µL of AAPH were pipetted into each well. Fluorescent measurements were then taken every 2 minutes for approximately 2 hours at an excitation wavelength of 485nm and an emissions wavelength of 520nm.

The AUC and Net AUC values of the standards and samples were determined using Gen5 2.0 Data Reduction Software using the below equations:

$$AUC = 0.5 + \frac{R2}{R1} + \frac{R3}{R1} + \frac{R4}{R1} + \cdots + \frac{Rn}{R1} \rightarrow Where\ R\ is\ fluorescence\ reading$$

$$Net\ AUC = AUC_{sample} - AUC_{blank}$$

The standard curve was obtained by plotting the Net AUC of different Trolox® concentrations against their concentration. ORAC values of samples were then calculated automatically using the Gen5 software to interpolate the sample's Net AUC values against the Trolox® standard curve. ORAC measurements for the test material were expressed in micro moles Trolox® equivalents per mL (µMTE), where 1 ORAC unit is equal to 1 µMTE.

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Materials

A. Kit:	CellROX™ Orange Reagent (ThermoFisher Scientific, C10443)
B. Incubation Conditions:	37°C at 5% CO_2 and 95% Relative Humidity (RH)
C. Equipment:	Forma humidified incubator; ESCO biosafety laminar flow hood; Light microscope; Synergy HT Microplate Reader; Pipettes
D. Cell Line:	Normal Human Neo-Natal Dermal Primary Fibroblasts (ATCC PCS-201-010)
E. Media/Buffers:	Fibroblast Basal Medium (PCS-201-030); Fibroblast Growth Kit (PCS-201-041); Ethanol; Phosphate Buffered Saline (PBS)
F. Reagents:	Hoechst 33342 (ThermoFisher Scientific, 62249); Antimycin A (Sigma Aldrich, A8674)
G. Culture Plate:	96 Well Black Side/Clear Bottom Tissue Culture Treated Microplates
H. Other:	Sterile disposable pipette tips

Methods

Human dermal fibroblasts were seeded into a 96-well tissue culture microplate and grown to 80%-90% confluency in complete media (CM). 0.01%, 0.1% and 1.0% concentrations of **ACB Pisum Sativum Peptide** in CM were added to cells and placed at 37°C. Control wells were incubated with CM only. Following an 18-hour incubation, the media in all wells was removed and cells were washed once with PBS. Hoechst and CellROX™ Orange were diluted in CM, and added to all wells at final concentrations of 20 µM and 5 µM, respectively. Following a 30-minute incubation at 37°C, the Hoechst and CellROX™ Orange solution was removed and cells were washed once with PBS. Next, 200 pM of Antimycin A (AntA), initially dissolved in ethanol and further diluted in CM, was added to all wells, except control wells that received CM. Following another 30-minute incubation at 37°C, the AntA and CM was removed, CM was added to all wells, and fluorescence measurements were taken with the following wavelengths (excitation / emission): Hoechst (361 nm / 486 nm) and CellROX™ Orange (545 nm / 565 nm).

To account for differences in cell counts, ROS levels are expressed as the ROS Signal (CellROX™ Orange) divided by the Nuclear Signal (Hoechst), as calculated by the following equation:

$$ROS\ Levels = \frac{ROS\ Signal}{Nuclear\ Signal}$$

Percent change is expressed relative to AntA and calculated by the following equation:

$$Percent\ Change\ (\%) = \frac{RFU_{Sample} - RFU_{AntA}}{RFU_{AntA}} \times 100$$

Salon Half-Head Study

MATERIALS AND METHODS

The study was conducted using five participants. Each subject had their baseline photo taken prior to having their hair washed. The participant was also asked to complete a survey rating their hair prior to treatment on a scale of 1 to 10, with 1 being the lowest and 10 being the highest, using the following parameters cleansing, smoothing, dry and wet combability, anti-frizz, overall feel, shine and hydration.

Half of the head was treated with the control shampoo and conditioner while the other half of the head was treated with 2.0% **ACB Pisum Sativum Peptide** in the base shampoo and base conditioner. After the application and rinse of the test and positive control products, each participant's hair was blown dry using a round brush on both sides of the head. Once the hair was completely dry, the participant was asked to again assess the same parameters of both halves of their hair. Assessments were made using a rubric from 1 to 10, with 1 being the lowest and 10 being the highest.

RESULTS

Parameters Tested	Assessment of the Control Shampoo	Assessment of the Experimental (2.0% ACB Pisum Sativum Peptide in Control Shampoo)	Assessment of the Control Conditioner	Assessment of the Experimental (2.0% ACB Pisum Sativum Peptide in Control Conditioner)
Cleansing	**6.25**	**7.25**	x	x
Smoothing	**6.14**	**8.33**	6.33	9.25
Wet Combability	**3.33**	**5.25**	6.00	9.15
Dry Combability	X	X	6.10	8.33
Anti-Frizz	X	X	5.22	8.46
Overall Feel	X	X	7.00	9.50
Shine	X	X	7.50	9.82
Hydration	X	X	8.25	9.70
Mean	**5.24**	**6.94**	**6.165**	**9.2**

Chart 1. Average Results for Participant's Sensory Assessment .

Parameters Tested	Percent Difference – Comparison of Control Shampoo vs. Experimental (2.0% ACB Pisum Sativum Peptide in Control Conditioner Shampoo)	Percent Difference – Comparison of Control Conditioner vs. Experimental (2.0% ACB Pisum Sativum Peptide in Control Conditioner)
Cleansing	14.81%	x
Smoothing	30.26%	37.48%
Wet Combability	44.75%	41.58%
Dry Combability	x	30.90%
Anti-Frizz	x	47.36%
Overall Feel	x	30.30%
Shine	x	26.70%
Hydration	x	16.15%

Chart 2. Percent Difference of Participant's Sensory Assessment.

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Materials

A. Kit:	Sirius Red/Fast Green Collagen Kit (Chondrex; 9046)
B. Incubation Conditions:	37°C at 5% CO_2 and 95% Relative Humidity (RH)
C. Equipment:	Forma Humidified Incubator, ESCO Biosafety Laminar Flow Hood, Synergy HT Microplate Reader; Pipettes
D. Cell Line:	Normal Human Dermal Fibroblasts (Invitrogen; C-004-5C)
E. Media/Buffers:	Complete and Serum-Free Dulbecco's Modified Eagle Medium (DMEM); Phosphate Buffered Saline (PBS)
F. Culture Plate:	Falcon Flat Bottom 24-Well Tissue Culture Treated Plates
G. Reagents:	Ascorbic Acid-2-Glucose(AA2G) (100µM); Insulin Growth Factor-1 (IGF-1) (50ng/mL); Glacial Acetic Acid, Ethanol
H. Other:	Sterile Disposable Pipette Tips; Wash Bottles

Methods

Human dermal fibroblasts were seeded into 24-well tissue culture plates and allowed to grow to confluency in complete DMEM. 1%, 0.1%, and 0.01% concentrations of **ACB Pisum Sativum Peptide** were added to the serum-free DMEM and incubated with fibroblasts for 24 hours. AA2G and IGF-1 were used a positive controls.

Media was removed from wells containing adherent fibroblasts and the cells were washed with PBS. 500µl of a cooled 95% ethanol/5% glacial acetic acid solution was added to the wells and incubated for 10 minutes at room temperature. 200µL of the Sirius Red/Fast Green dye solution was added to the fixed cell layer and incubated at room temperature for 30 minutes. The dye solution was removed from the cell layer and washed with water. 1mL of extraction solution was added for color extraction. The optical density was read at 540nm and 605nm on the Synergy HT Microplate Reader.

The protein concentrations of **ACB Pisum Sativum Peptide** treated-fibroblasts were determined by calculations based on the optical density measurements and expressed in µg.

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Assay Principle

This ELISA utilizes a colorimetric reaction employing antibodies with antigen specificity to Human/Mouse TGF-β1. The Human/Mouse TGF-β1 ELISA recognizes the mature/active form of TGF-β1 without association with Latency Associated Peptide (LAP). The samples require acid-treatment and neutralization to remove LAP from TGF-β1 prior to evaluation. Antibodies specific for TGF-β1 epitopes are coated on a microtiter plate. In positive samples, TGF-β will bind to these antibodies and are tagged a second time with another TGF-β-specific antibody labeled with horseradish peroxidase (HRP). The addition of the chromagen/substrate solution, containing 3,3',5,5'-tetramethylbenzidine, provides the colorimetric reaction with HRP that is quantitated through optical density (OD) readings on a microplate spectrometer. The standard curve provides a reference from the OD readings for the amount of TGF-β1 in each sample.

Materials

A. Kit:	Human/Mouse TGF-β 1 ELISA Kit (eBioscience; 88-8350)
B. Incubation Conditions:	37°C at 5% CO_2 and 95% relative humidity (RH)
C. Equipment:	Forma humidified incubator; ESCO biosafety laminar flow hood; Microplate Reader; Pipettes; Centrifuge
D. Cell Line:	Normal Human Dermal Fibroblasts (NHDF) (Lonza; CC-2511)
E. Media/Buffers:	Basal Medium (Fibrolife; LM-0001), 500µg/mL Human Serum Albumins (Fibrolife; LS-1001), 0.6µM Linoleic Acid (Fibrolife; LS-1001), 0.6µg/mL (Fibrolife; LS-1001), 5ng/mL Fibroblast Growth Factor (Fibrolife; LS-1002), 5mg/mL Epidermal Growth Factor (Fibrolife; LS-1003), 30pg/mL Transforming Growth Factor β-1 (Fibrolife; LS-2003), 7.5mM L-Glutamine (Fibrolife; LS-1006), 1µg/mL Hydrocortisone Hemisuccinate (Fibrolife; LS-1007), 50µg/mL Ascorbic Acid (Fibrolife; LS-1005), 5µg/mL Insulin (Fibrolife; LS-1004)
F. Culture Plate:	Falcon flat bottom 24-well tissue culture treated plates
G. Reagents:	500X Cell Stimulation Cocktail (eBioscience; 00-4970-93); Pirfendione (Sigma Aldrich; P2116-10MG); Hydrochloric Acid (HCL); Sodium Hydroxide (NaOH)
H. Other:	Sterile disposable pipette tips; wash bottles

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Results

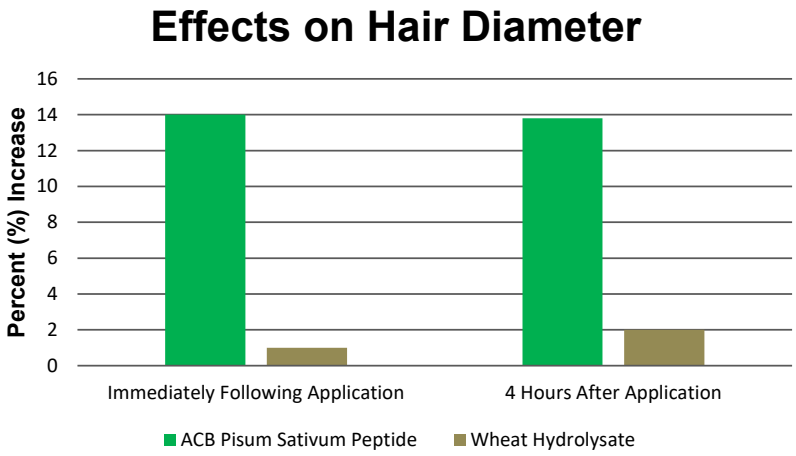

Figure 1. Percent increase in hair diameter over time after treatment with **ACB Pisum Sativum Peptide** and Wheat Hydrolysate

Microscopy Imaging of Individual Hair Strands

Figure 2: Individual strand immediately following treatment with wheat Hydrolysate (note beading)

Figure 3: Individual strand immediately following treatment with **ACB Pisum Sativum Peptide**