Application of Collagen Hydrolysate Extracted from Limed Hide Waste in the Crop Grow Seedlings

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Summary: In this work, the collagen hydrolysates with different molecular weights were successfully extracted from the limed hide waste, and used at the nutrient solutions for crop breeding. Firstly, using the single factor and orthogonal exp eriments, hydrolytic process parameters of limed hide waste were optimized for the Alcalase-based enzymatic and $Ca(OH)_2$-based alkali, and alkali-enzyme methods that led to the corresponding collagen hydrolysates with different molecular weights. The obtained collagen hydrolysates were characterized by gel permeation chromatography (GPC), amino acid analysis, the inductively coupled plasma optical emission spectrometer (ICP-OES), and Kjeldahl method. Then, the collagen hydrolysates were used as organic nitrogen sources to prepare fertilizers for the grow seedlings of *Triticum aestivum*, *Glycine max*, and *Brassica napus*, and inorganic nitrogen solutions were used as controls. The effects of these nutrient solutions on the seedlings and growth of the three crops were investigated. Concretely, the germination rate, plant weight, seeding height, soluble sugar content, and chlorophyll content were tested. Results indicated that all the collagen hydrolysate products could be used to prepare water-soluble fertilizers that can intensively boost germination, plant weight, and seeding height, and greatly increase soluble sugar and chlorophyll content in leaves. Furthermore, the fertilizer efficiencies of all the tested degradation products are much better than that of the water-soluble fertilizer containing inorganic nitrogen. All the proteolytic nutrient solutions with different molecular weights can promote the growth of crops, but the smaller the molecular weight is, the better the growth effect of crops is, which is manifested in the higher germination rate, plant weight, seedling height, soluble sugar content and chlorophyll content of seeds.

Keywords: Leather industry; Limed hide waste; Collagen hydrolysate; Crop; Seedlings.

Introduction

Excessive application of chemical fertilizers not only affects the quality of agricultural products but also damages the soil ecological environment [1-4]. It is very important to develop high-efficiency and pollution-free green fertilizers (or nutrients) to satisfy the production of organic food. As organic nitrogen and carbon resources, amino acids can be directly absorbed by plants without being transformed, which can improve the quality and yield of plants [5,6], increases the protein and sugar content of crops, enhances the resistance of plants [7,8], removes the active oxygen free radicals in the plant body[10], and reduces the nitrate content in plants[10]. In addition to providing nitrogen for plants, amino acids can also improve the quality of soil, reduce the residue of fertilizers in the soil, and modify the environmental pollution. Amino acids have no side effect on the environment and humans and are good raw material for fertilizers.

Hydrolyzed protein, containing a large number of amino acids, can improve plant growth very well[11]. There are abundant protein resources that can be used to produce amino acid fertilizers. Among them, there is a large amount of leather protein wastes, and their protein contents are high. It has a good application prospect to prepare amino acid fertilizers by using the hydrolyzed collagen extracted from the leather solid wastes. For example, Liu et al [12] prepared amino acid fertilizers using chrome shavings. The collagen hydrolysate with high molecular weight was firstly extracted from chrome shavings by alkaline hydrolysis and then hydrolyzed into small peptides and amino acids by acid hydrolysis. The free amino acid content in the obtained fertilizer was determined to be 32.4%, which is 223.8% higher than the industrial technical index. Chen et al [13] reported the preparation of functional organic fertilizer by using chrome shaving as raw material, and its efficiency to improve the

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quality and yield of buckwheat was confirmed. The utilization efficiency of nitrogen element in buckwheat was increased, and the content of the flavonoid compounds was also improved by using this fertilizer. Epure et al [14] prepared the collagen hydrolysate by the alkaline-enzymatic hydrolysis of wet-white shavings and studied its influence on the cereal (wheat, barley, rye, oats) seed growth stimulation and crop production improvement. It was found that the use of collagen hydrolysate could lead to the increase of germination rate and energy of germination rate. Compared to the untreated seeds, the seeds treated with collagen hydrolysate had high levels of gibberellic acid content, and the corresponding seedling had high biomass. However, there is no report about the application of collagen hydrolysate extracted from limed hide solid waste in the crop breeding cultivating.

The current studies suggested that the smaller the molecular weight of the hydrolysis product of leather waste is, the better the absorption by plants [15-18], but there is no direct test to prove this hypothesis. Furthermore, there is no report about the comparison of amino acid fertilizer on the stimulant effect on plants of different families and genera. For this purpose, in this paper, we investigated the effects of collagen hydrolysate with different molecular weights extracted from cattle hide solid waste on the germination and growth of three crops with different families and genera (*Triticum aestivum*, *Glycine max*, and *Brassica napus*). We recorded the growth state (e.g. height and weight), chlorophyll, and soluble sugar contents of three crops to explore the promoting effect of collagen hydrolysate with different molecular weights. In this way, the optimum molecular weight of collagen hydrolysate can be found to maximize the utilization of limed hide solid waste and provide better methods and resources for amino acid fertilizers.

Experimental

Materials

Limed cattle hide solid waste (LCHSW) was provided by the experimental plant in the department of biomass and leather engineering. Alcalase enzyme and hydroxyproline were purchased from Saen Chemical Technology Co. LTD. The other chemicals used in this work were from commercial channels and used as directly.

Pretreatment of LCHSW

The LCHSW was weighed as the reference for the dosage of the following materials and then stirred for 60 min in water (80%) at room temperature (RT, 25 ^{o}C or so) in the presence of ammonium chloride (2%) and hydrochloric acid (2%). After that, the pH of the solution was adjusted to 6.0-7.0, and the resulting LCHSW was taken out and cut into pieces (0.5 cm × 0.5 cm) for the following experiments.

Analysis of LCHSW pieces

The moisture content of pretreated LCHSW pieces was determined by using the drying method following the standard procedure of GB5009.3−2016[19]. The ash content was determined by using the method of burning of high temperature described in GB5009.4−2016[20]. The protein content was determined by using the Kjeldahl method provided in GB5009.5−2016[21]. The fat content was measured by using the Soxhlet extraction method described in GB5009.6−2016[22].

General enzyme extraction procedure

The pretreated LCHSW pieces (2.0 g) were added into the aqueous solution of Alcalase (50 mL), and the obtained mixture was stirred at a certain temperature for a period of time. The filter operation was then conducted by using the cotton gauze, and the filtrate was collected and centrifuged for 10 min at the rotate speed of 8000 r/min. The resulting supernatant liquor was collected and lyophilized in a vacuum freezer dryer (LGJ-10N, Beijing Yaxing Technology Development Co. LTD) to provide the final enzymatic collagen hydrolysate (ECH) product. The tested temperatures were in the range of 55-85 ^{o}C, and the treatment time was adjusted in the period of 1-6 h. The dosage of Alcalase was based on the weight of LCHSW pieces and was controlled in the scope of 2%-8%.

General alkali extraction procedure

The pretreated LCHSW pieces (2.0 g) were added into the aqueous solution of $Ca(OH)_2$ (50 mL), and the obtained mixture was stirred at a certain temperature for a period of time. After that, the pH was adjusted to 7.0 by using hydrochloric acid (HCl). The filter operation was then conducted by using the cotton gauze, and the filtrate was collected and centrifuged for 10 min at the rotate speed of 8000 r/min. The resulting supernatant liquor was collected and lyophilized to give the final alkali collagen hydrolysate (ACH) product. The tested temperatures

were in the range of 75-115 °C, and the treatment time was adjusted in the period of 2-8 h. The dosage of $Ca(OH)_2$ was based on the weight of LCHSW pieces and was controlled in the scope of 7%-13%.

Alkali-enzyme extraction procedure

The pretreated LCHSW pieces (2.0 g) were added into the aqueous solution of $Ca(OH)_2$ (50 mL, 12%), and the obtained mixture was stirred at 110 °C for 7 h. After that, the mixture was cooled to room temperature, and its pH was adjusted to 7.5 by using the HCl solution. Alcalase (4%) was then added into the mixture and stirred at 75 °C for 2.5 h. Subsequently, the filter operation was conducted by using the cotton gauze, and the filtrate was collected and centrifuged for 10 min at the rotate speed of 8000 r/min. The resulting supernatant liquor was collected and lyophilized to give the final alkali-enzymic collagen hydrolysate (AECH) product.

Analysis of the extracted protein hydrolysate

Hyp content: The lyophilized protein hydrolysate (0.05 g) was dissolved in the HCl solution (250 mL, 0.01 mol/L). The obtained solution was treated by using the reported standard procedure[23], and its absorbance at 560 nm was recorded by using a UV-vis spectrophotometer (UV1900, Shanghai Aoyi Instrument Co., LTD). The hydroxyproline (Hyp) content in the protein hydrolysate was calculated by using the standard curve of Hyp.

Protein content: The Kjeldahl method was adopted to determine the protein content in the lyophilized protein hydrolysate.

Molecular weight: The gel permeation chromatography (GPC) was used to determine the molecular weight of the lyophilized protein hydrolysate. The GPC measurements were conducted in water using Shimadzu high performance liquid chromatography (HPLC) system equipped with PLgel 5 μm MIXED-D columns, refractometric and UV detectors, column oven, and integrated degasser. Molecular weights were calculated based on the multiangle light scattering data using the Wyatt Astra software, with dn/dc values of the polymers determined from the RI detector using Astra. Column calibration was performed using polyethylene glycol (PEG) standards from Polymer Laboratories.

Metal content: The decomposition of the protein hydrolysate was conducted using the mixture of nitric acid and perchloric acid, and the metal (As, Cd, Cr, Pb, etc.) contents were determined by using the inductively coupled plasma optical emission spectrometer (ICP-OES). The content of Hg was determined by using the atom fluorescence spectrum method.

Preparation of culture solutions for seeds

The formulations of the culture solutions for seeds were listed in Table S1. The concentrations of nitrogen were controlled to be 10 mg/L. The blank group is composed of deionized water, while the control group contains inorganic salts.

General seed breeding procedure

50 seeds (*Triticum aestivum*, *Glycine max*, and *Brassica napus*) were evenly distributed on the filter paper in a container (beaker or petri dish). 3 mL of culture solution were evenly sprayed on the surfaces of these seeds. This spraying treatment was repeated two times every day. After 2 days, the germination rate of seeds was determined. After 16 days (or 17 days for *Glycine max* and 30 days for *Brassica napus*), the height and weight of seedlings were determined, and their chlorophyll contents[24] and soluble sugar contents[25] were measured using the reported methods.

Results and Discussion

Characterization of collagen hydrolysates with different molecular weights

The limed cattle hide solid waste (LCHSW) used in this work was analyzed, and the results were listed in Table S1. The protein content was determined to be 72.20%, indicating the LCHSW is a good amino acid resource. The optimal experiments were then conducted to give the optimizing process conditions used to extract the collagen hydrolysate from the LCHSW. For the enzyme extraction procedure, Alcalase was used and the single factor experiments were carried out to optimize its reaction temperature, time, and dosage. Fig.S1 shows the effect of treatment temperature (55-85 °C) on the hydroxyproline (Hyp) concentration of the resulting enzymatic collagen hydrolysate (ECH) when the treatment time was 2 h and the dosage of Alcalase was 10%. It can be seen that the Hyp content in the ECH increases with the rise of temperature in the range of 55-75 °C. When the temperature reaches 75

°C, the Hyp content reaches the maximum value. As the reaction temperature continues to increase, Hyp content decreases. These results could be explained by the temperature sensibility of the catalytic activity of Alcalase. When the temperature was less than 75 °C, the activity of Alcalase was gradually activated with the increase of temperature. When the temperature exceeds this temperature, the activity of the enzyme begins to decrease and the enzyme begins to denature, which reduces its hydrolysis ability of the collagen in limed hide and thus leads to the decrease of Hyp content in the hydrolyzed products. Based on the above results, 75 °C was selected as the enzymatic hydrolysis temperature of LCHSW for the next experiment. Similarly, the reaction time was optimized to be 2 h (Fig.S2) when the dosage of Alcalase was 10% and the temperature was 75 °C, and the dosage of Alcalase was optimized to be 4% (Fig.S3) when the treatment temperature and time were 75 °C and 2 h, respectively. According to the results of the above single factor experiments, we further optimize the conditions of the enzymatic hydrolysis of LCHSW using the orthogonal design as shown in Table S2, and the orthogonal experimental results were listed in Table S3. It can be seen that the extreme value order of the analysis results is Y > X > Z. Namely, among the three conditions, the reaction temperature has the greatest influence on the effect of Alcalase hydrolysis, followed by reaction time and finally the amount of Alcalase. The best conditions are as follows: 70 °C, 2.5 h, and 4% dosage of Alcalase. Under these optimal conditions, the Hyp content in the final ECH product could reach 55.62 mg/g.

For the alkali extraction procedure, $Ca(OH)_2$ was used and the single factor experiments were also carried out to optimize its reaction temperature (Fig. S4), time (Fig. S5), and dosage (Fig. S6). The orthogonal experiments were further designed (Table S4), and the corresponding results were listed in Table S5. It can be seen that the extreme value order of the analysis results is Y > X > Z. That is, the reaction temperature has the greatest influence on the effect of $Ca(OH)_2$ hydrolysis, followed by reaction time and finally the amount of $Ca(OH)_2$. The best conditions are as following: 110 °C, 7 h, and 12% dosage of Alcalase. Under these optimal conditions, the Hyp content in the final alkali collagen hydrolysate (ACH) product could reach 56.98 mg/g.

The alkali-enzyme extract method was carried using the above optimization results for enzyme and alkali extraction procedures to obtain the final alkali-enzymic collagen hydrolysate (AECH) product with Hyp content of 59.25 mg/g.

Table-1 provides the component analysis results of the extracted collagen hydrolysate. The protein contents are 88.9% for ECH, 83.3% for ACH, and 93.8% for AECH. These results indicate that the alkali-enzymic method can result in the collagen hydrolysate with the highest protein content. Moreover, the common elements were detected by using the inductively coupled plasma optical emission spectrometer (ICP-OES). As can be seen in Table 1, for the ACH product, the most abundant element is sulfur (S) whose content is 2449.7 mg/kg. The sulfur element is resulted from the sulfur-containing amino acids (e.g. methionine and cysteine). As expected, there is high content of calcium (Ca) element, which is attributed to the use of $Ca(OH)_2$ for the limed hide. Furthermore, silicon (Si), sodium (Na), phosphorus (P), ferrum (Fe), magnesium (Mg), and potassium (K) elements were also detected with high contents. These elements are beneficial for the growth of plants, so the collagen hydrolysate have the potential to be used as fertilizer. Notably, there are no detectable toxic heavy metals such as arsenic (As), chromium (Cr), mercury (Hg), lead (Pb), and cadmium (Cd). Compared to the ECH, the ACH product contains similar elements, but there are differences in the contents of elements. For example, owing to the use of $Ca(OH)_2$ during the period of hydrolysis, the Ca content of ACH is nearly 10 times that of ACH. The contents of S, Na, and K are also improved, while the contents of Si, P, Fe, and Mg are slightly decreased. Furthermore, there is a very small amount of aluminum (Al) and strontium (Sr) in ACH, which is probably resulted from the used $Ca(OH)_2$. For the AECH product, the similar elemental composition is observed. There are also high contents of Ca, S, Na, and Si, and moderate contents of K, Fe, and P.

Table 2 shows the amino acid analysis results of the extracted collagen hydrolysate. It can be seen that there are 21 kinds of amino acids for every collagen hydrolysate, but their contents show a slight difference in the three products. There is no asparagine, citrulline, and methionine. The most abundant amino acids are glycine, proline, glutamic acid, hydroxyproline, alanine, arginine, aspartic acid, cysteine, etc. Notably, for each kind of amino acid, the AECH product contained moderate higher content than the ECH and ACH product, which could be attributed to their different preparation methods and molecular weights.

Table-1: Component analysis of the extracted collagen hydrolysate.

Parameter	ECH	ACH	AECH	Parameter	ECH	ACH	AECH
Ash (%)	0.9	0.9	0.9	Ca (mg/kg)	504.5	49758.0	36785.6
Protein (%)	88.9	83.3	93.8	Si (mg/kg)	601.6	281.4	304.4
Mn (mg/kg)	5.6	<5.0	18.1	S (mg/kg)	2449.7	2626.4	2832.7
Zn (mg/kg)	6.3	<5.0	<5.0	Al (mg/kg)	<5.0	11.3	14.4
B (mg/kg)	8.9	10.5	17.2	Sr (mg/kg)	<5.0	15.7	14.7
Ni (mg/kg)	10.8	<5.0	<5.0	As (mg/kg)	<5.0	<5.0	<5.0
Sn (mg/kg)	29.8	20.3	11.7	Cr (mg/kg)	<5.0	<5.0	<5.0
K (mg/kg)	40.7	105.0	83.9	Hg (mg/kg)	<5.0	<5.0	<5.0
Mg (mg/kg)	57.6	31.2	<5.0	Pb (mg/kg)	<5.0	<5.0	<5.0
Fe (mg/kg)	58.0	41.6	55.0	Cd (mg/kg)	<5.0	<5.0	<5.0
P (mg/kg)	101.1	75.5	35.9	Cu (mg/kg)	<5.0	<5.0	<5.0
Na (mg/kg)	253.9	643.6	435.8	Mo (mg/kg)	<5.0	<5.0	<5.0

Table-2: Amino acid content of the extracted collagen hydrolysate (mg/g).

Type	ECH	ACH	AECH	Type	ECH	ACH	AECH
Aspartic acid	24.167	23.012	26.620	Cysteine	19.348	17.647	22.196
Glutamic acid	41.327	38.728	45.967	Valine	14.733	14.087	15.634
Asparagine	0.000	0.000	0.000	Methionine	0.000	0.000	0.000
Serine	11.013	10.819	15.153	Norvaline	3.730	3.928	4.950
Glutamine	0.000	0.000	0.679	Tryptophan	5.966	5.760	7.583
Histidine	4.189	2.992	3.246	Phenylalanine	10.515	11.076	13.052
Glycine	71.807	66.712	79.912	Isoleucine	7.613	7.797	10.400
Threonine	6.810	7.492	10.885	Leucine	13.095	12.811	14.671
Citrulline	0.000	0.000	0.000	Lysine	11.438	11.880	15.265
Arginine	24.827	22.754	32.915	Hydroxyproline	34.710	30.220	39.838
Alanine	32.464	29.989	37.087	Sarcosine	0.190	0.275	0.242
Tyrosine	3.651	3.920	4.114	Proline	47.983	38.908	58.579

The gel permeation chromatography (GPC) was used to determine the molecular weight of the extracted collagen hydrolysates, and the results are shown in Fig. 1. All three GPC curves show singlet broad peaks, indicating the wide distribution of molecular weights for the three collagen hydrolysates. Using polyethylene glycol (PEG) as the standard, the relative molecular weights were calculated and listed in Table 3. Concretely, the weight-average relative molecular weights (M_w) of ECH, ACH, and AECH were 1373 Da, 1913 Da, and 1245 Da, respectively, while the numerical-average relative molecular weights (M_n) were 926 Da for ECH, 1130 Da for ACH and 734 Da for AECH. Obviously, the ECH product has the highest molecular weight, while the AECH possesses the lowest molecular weight. Furthermore, the polydispersity indexes (PDI) were calculated by comparing the values of M_w and M_n. The GPC curve of the ECH product exhibits the lowest PDI value of 1.48, while the GPC curves of ACH and AECH products show a similar PDI of 1.70 or so. The former is probably resulted from the specific hydrolysis of Alcalase, while the latter could be explained by the undifferentiated decomposition of $Ca(OH)_2$.

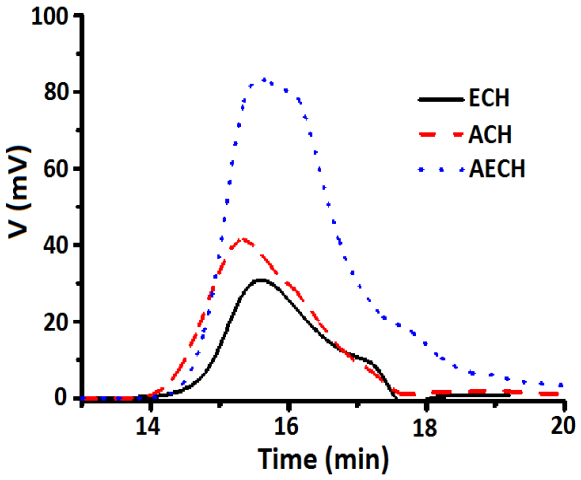

Fig. 1: GPC curves of the three extracted collagen hydrolysates.

Table-3: Molecular weights by GPC of the three extracted collagen hydrolysates.

Sample	M_n	M_w	M_z	M_η	PDI (M_w/M_n)
ECH	926	1373	1902	1296	1.48
ACH	1130	1913	2925	3983	1.69
AECH	734	1245	1883	1154	1.70

Seed culture effect of collagen hydrolysates

The formulations of the culture solutions for seeds were listed in Table 4. The concentrations of nitrogen were controlled to be 10 mg/L. The blank group is composed of deionized water, while the control group contains inorganic salts. The culture solutions prepared by the ECH, ACH, and AECH products are named as B-1, B-2, and B-3, respectively.

Table-4: Formulations of the culture solutions for seeds.

Component	Blank	Control	B-1	B-2	B-3
Hydrolysate (g/L)	-	-	0.933	1.050	0.984
NH_4Cl (g/L)	-	0.535	-	-	-
K_2SO_4 (g/L)	-	0.350	0.350	0.350	0.350
KH_2PO_4 (g/L)	-	0.270	0.270	0.270	0.270
$CaCl_2$ (g/L)	0.160	0.160	0.160	-	-

As shown in Table 5, the germination rates are 86% for the B-1 group, 84% for the B-2 group, and 90% for the B-3 group. All these values are higher than the germination rates of the blank group, indicating the stimulating action of collagen hydrolysate on the germinating of *Triticum aestivum* seeds. Notably, the B-3 group, in which the molecular weight of collagen hydrolysate is the lowest, exhibited the highest germination rate. The germination rate of the control group is 84%, and equal to that of the B-2 group with the biggest molecular weight of collagen hydrolysate, but lower to those of the B-1 and B-3 groups. After 16 days of culture, the height and weight of seedlings were determined. As shown in Table 5, as expected, the B-3 group possesses the heaviest and tallest seedlings, and the corresponding values are 0.126 g and 21.04 ± 1.6 cm, respectively. For the B-1 and B-2 groups, their weights of seedlings are 0.121 g and 0.117 g, and their heights of seedlings are 20.12 ± 1.7 cm and 19.81 ± 1.1 cm. All these values are bigger than those of the blank and control groups. The better growth situations of the test groups were also confirmed by the photographs shown in Fig. 2. Notably, compared to the blank and control groups, the B-1, B-2, and B-3 groups show the seedlings with deeper green color. All these results indicate that the collagen hydrolysates extracted from the limed hide are good fertilizer to promote the germination and growth of *Triticum aestivum*. Their promotion effects are better than the control group containing the inorganic salts. More importantly, it is obvious that the molecular weight of collagen hydrolysate could affect fertilizer efficiency. The smaller the molecular weight of collagen hydrolysate is, the better the effect of promoting seed germination and seedling growth.

Table-5: Germination rate, height and weight of seedlings.

	Sample	Germination rate/%	Weight/g	Height/cm
Triticum aestivum	Blank	82	0.103	18.21±1.2
	Control	84	0.108	19.33±1.6
	B-1	86	0.121	20.12±1.7
	B-2	84	0.117	19.81±1.1
	B-3	90	0.126	21.04±1.6
Glycine max	Blank	80	0.631	26.30±1.2
	Control	84	0.646	28.20±1.7
	B-1	90	0.667	31.50±1.6
	B-2	88	0.663	30.50±2.2
	B-3	92	0.681	33.50±2.5
Brassica napus	Blank	36	0.024	3.50±1.2
	Control	38	0.032	4.50±1.0
	B-1	42	0.038	6.10±1.2
	B-2	38	0.034	4.70±1.8
	B-3	48	0.041	6.80±1.6

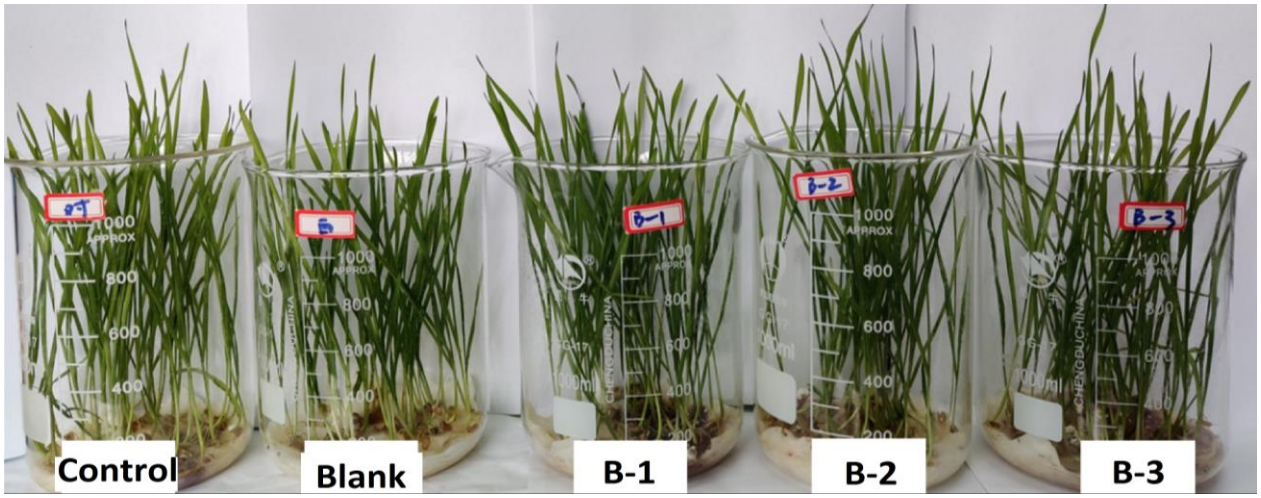

Fig. 2: Photographs of *Triticum aestivum* after 16 days of culture.

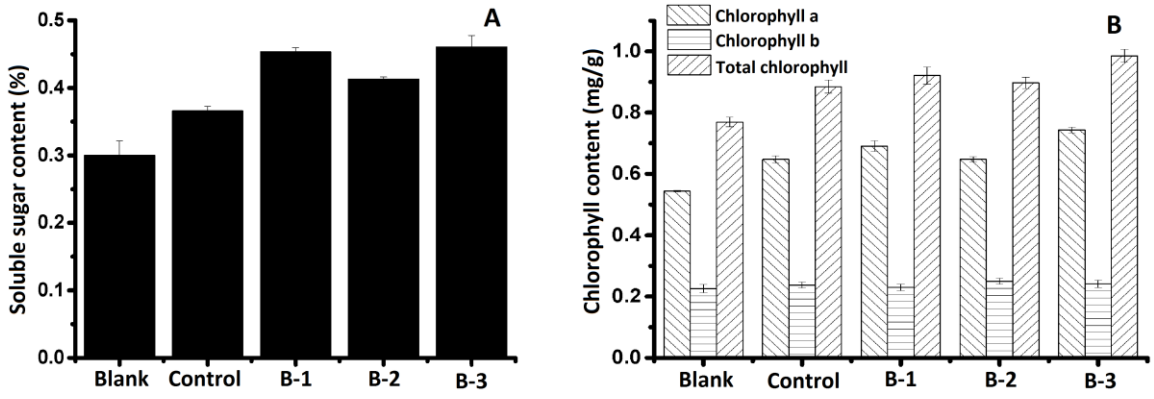

Fig. 3: Soluble sugar (A) and chlorophyll (B) contents of *Triticum aestivum* seedlings.

Fig. 3A provides the soluble sugar contents of *Triticum aestivum* seedlings. As can be seen that, the soluble sugar contents of *Triticum aestivum* seedlings cultivated by nutrient solution prepared by collagen hydrolysate in the three groups were higher than that of the blank and control groups, and the sequence was as follows: B-3 > B-1 > B-2 > control group > blank group. The soluble sugar content in the B-3 group was 0.46%, which was 0.16%, 0.09%, 0.0072%, and 0.048% higher than that in the blank group, the control group, the B-1 group, and the B-2 group, respectively, and the gap with the blank group was particularly obvious. These results indicated that the amino acid fertilizers prepared by collagen hydrolysate from limed hides could promote the production of soluble sugar in *Triticum aestivum*, and the smaller the molecular weight was, the more obvious the promotion effect was. That is to say, the soluble sugar content in the B-3 group was the highest, which was also consistent with the growth status of *Triticum aestivum*, that is, the germination rate was the highest and the green color was deeper (Fig. 2).

Fig. 3B gives the chlorophyll a, chlorophyll b, and total chlorophyll contents of *Triticum aestivum* seedlings. It can be seen from the results that, after treated by the nutrient solution prepared by collagen hydrolysate with different molecular weights, chlorophyll a, chlorophyll b, and total chlorophyll in *Triticum aestivum* were all higher than that in the control and control groups. Especially, the B-3 group had the highest chlorophyll content, which was 0.74mg/g for chlorophyll a, 0.24mg/g chlorophyll b, and 0.98mg/g for total chlorophyll, respectively. Specifically, compared with the blank group, the chlorophyll a content in B-3 (the B-3) group was increased by 0.20 mg/g, the chlorophyll b content was improved by 0.01mg/g, and the total chlorophyll content was increased by 0.21mg/g. Compared with the B-2 group, the content of chlorophyll a in the B-3 group was 0.09 mg/g higher, the content of chlorophyll b was 0.008 mg/g lower, and the total content of chlorophyll was 0.082 mg/g higher. Compared with the B-1 group, the chlorophyll a content in the B-3 group was increased by 0.05 mg/g, the chlorophyll content was increased by 0.01 mg/g, and the total chlorophyll content was improved by 0.06mg/g. In short, the order for the content of chlorophyll is B-3 > B-1 > B-2 > control group > blank group. The amino acid fertilizers prepared by collagen hydrolysate from limed hides could promote the generation of chlorophyll in *Triticum aestivum* more than the blank and control groups. The smaller the molecular weight of the collagen hydrolysate, the more it can promote the formation of chlorophyll.

For *Glycine max*, similar experiments were also conducted to explore the culturing effect of amino acid fertilizers prepared by collagen hydrolysate from limed hides. As shown in Table 5, After 2 days of culture, the germination rates are 90% for the B-1 group, 88% for the B2 group, and 92% for the B-3 group. All these values are higher than the germination rates of the blank and control groups, indicating the stimulating action of collagen hydrolysate on the germinating of *Glycine max* seeds. Notably, the B-3 group, in which the molecular weight of collagen hydrolysate is the lowest, exhibited the highest germination rate. After 17 days of culture, the height and weight of seedlings were also determined. The better growth situations of the test groups were also confirmed by the photographs shown in Fig. 4. As shown in Table 5, after applying

the nutrient solution containing collagen hydrolysate, the weight and height of *Glycine max* seedlings in the tested groups were higher than those of the blank and control groups. And weight and height of seedlings in the B-3 group, which are 0.681 g and 33.5 ± 2.5 cm, are higher than those of the B-1 and B-2 groups. Specifically, compared with the blank group, the plant weight and seedling length of the B-3 group were increased by 0.05 g and 7.2 cm, respectively. Compared with the B-2 group, the weight and length of seedlings in the B-3 group were increased by 0.018 g and 3 cm, respectively. Compared with the B-1 group, the seedling weight and length of the B-3 group were increased by 0.014 g and 2 cm, respectively. The absorption effect of Glycine max on the amino acid nutrient solutions was better than that of the inorganic nitrogen control group, and the smaller the molecular weight was, the better the absorption effect and promotion effect were. Furthermore, the promotion effect of amino acid nutrient solutions on the germination rate of *Glycine max* was more obvious than that of *Triticum aestivum*.

Fig. 5A provides the soluble sugar contents of *Glycine max* seedlings. As can be seen that, the soluble sugar contents in the three test groups were significantly higher than that in the blank and control groups, and the sugar content (0.24%) in the B-3 group was the highest. Concretely, the soluble sugar content of *Glycine max* seedlings in the B-3 group was 0.11% higher than that of the blank group, 0.10% higher than that of the control group, 0.01% higher than that of the B-1 group, and 0.07% higher than that of B-2 group. The order of soluble sugar content is B-3 > B-1 > B-2 > control > blank. These results indicate that compared with the inorganic nitrogen control group, the amino acid groups could promote the production of soluble sugar in *Glycine max*, to promote the growth of *Glycine max*. It can be seen from the amino acid groups that the smaller the molecular weight is, the higher the soluble sugar content is. The collagen hydrolysate with low molecular weight was more easily absorbed by *Glycine max*, which promotes the production of soluble sugar and also improves the germination rate of *Glycine max*.

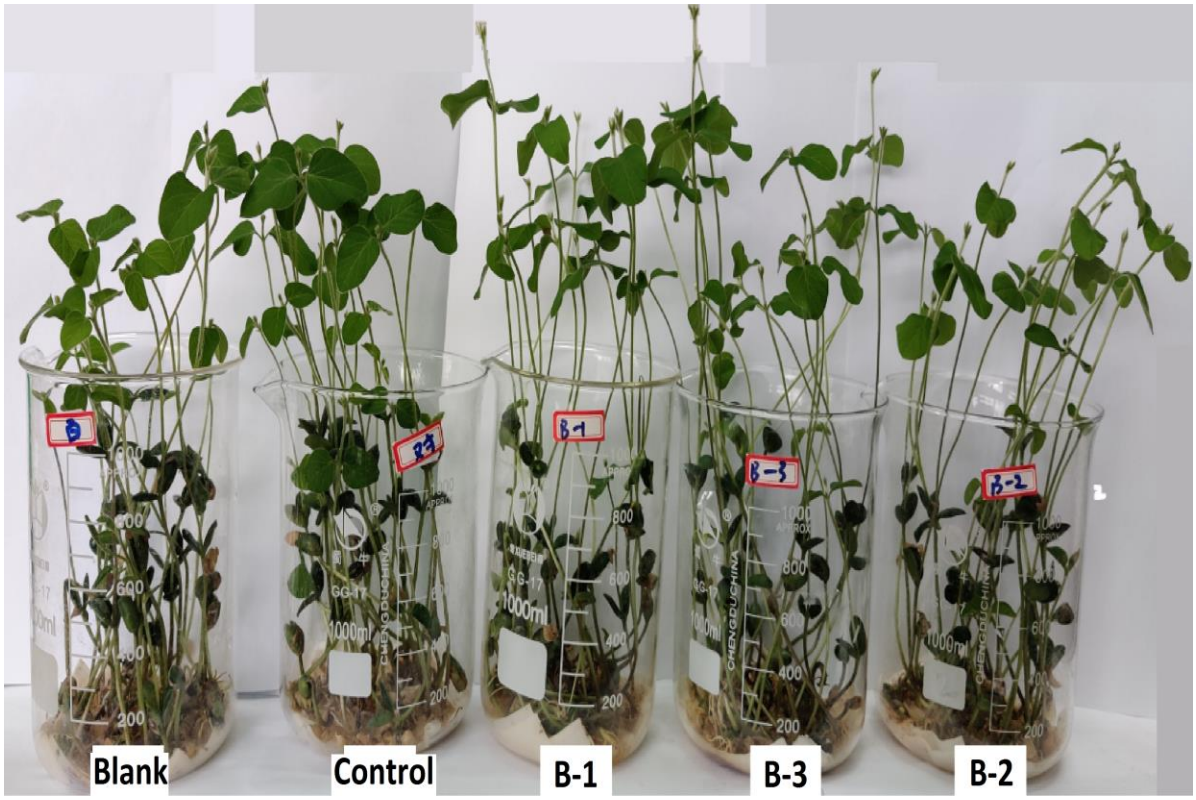

Fig. 4: Photographs of *Glycine max* after 17 days of culture.

Fig. 5B shows the chlorophyll a, chlorophyll b, and total chlorophyll contents of *Glycine max* seedlings. The chlorophyll a, chlorophyll b, and total chlorophyll contents of *Glycine max* seedlings in the B-3 group are 1.49 mg/g, 0.65 mg/g, and 2.14 mg/g, which were all higher than those of other groups. Specially, the increase amounts are 0.28 mg/g, 0.18 mg/g and 0.46 mg/g, respectively, compared with the blank group, 0.26 mg/g, 0.01 mg/g and 0.27 mg/g, respectively, compared with the control group, 0.063 mg/g, 0.001 mg/g and 0.064 mg/g, respectively, compared with B-1 group, and 0.094 mg/g, 0.064 mg/g and 0.158 mg/g, respectively, compared with B-2 group. The order of chlorophyll contents of *Glycine max* seedlings is B-3 > B-1 > B-2 > control > blank. These results indicate that the amino acid fertilizers containing collagen hydrolysate could promote the formation of chlorophyll in *Glycine max* leaves, and the smaller the molecular weight is, the stronger the promotion effect is.

For *Brassica napus*, similar experiments were also conducted to explore the culturing effect of amino acid fertilizers prepared by collagen hydrolysate from limed hides. As shown in Table 5, After 2 days of culture, the germination rates are 42% for the B-1 group, 38% for the B-2 group, and 48% for the B-3 group. All these values are higher than the germination rates of the blank group, indicating the stimulating action of collagen hydrolysate on the germinating of *Brassica napus* seeds. Notably, the B-3 group, in which the molecular weight of collagen hydrolysate is the lowest, exhibited the highest germination rate. The germination rate of the control group is 38%, and equal to that of the B-2 group with the biggest molecular weight of collagen hydrolysate, but lower to those of the B-1 and B-3 groups. However, compared with the *Triticum aestivum* and *Glycine max*, *Brassica napus*, exhibited greatly lower germination rates, which could be explained by the unfavorable breeding conditions provided by the experiments. After 30 days of culture, the height and weight of seedlings were determined. As shown in Table 5, as expected, the B-3 group possesses the heaviest and tallest seedlings, and the corresponding values are 0.041 g and 6.8 ± 1.6 cm, respectively. For the B-1 and B-2 groups, their weights of seedlings are 0.038 g and 0.034 g, and their heights of seedlings are 4.7 ± 1.8 cm and 6.1 ± 1.2 cm. All these values are bigger than those of the blank and control groups. The better growth situations of the test groups were also confirmed by the photographs shown in Fig. 6. It can be seen that the height and germination rate of *Brassica napus* seedlings cultured with the nutrient solution containing the collagen hydrolysate from limed hides were higher than that of the control and control groups, and the *Brassica napus* seedlings in group B-3 seemed to grow in the best state without any dumping, and the color difference could not be seen by the naked eye.

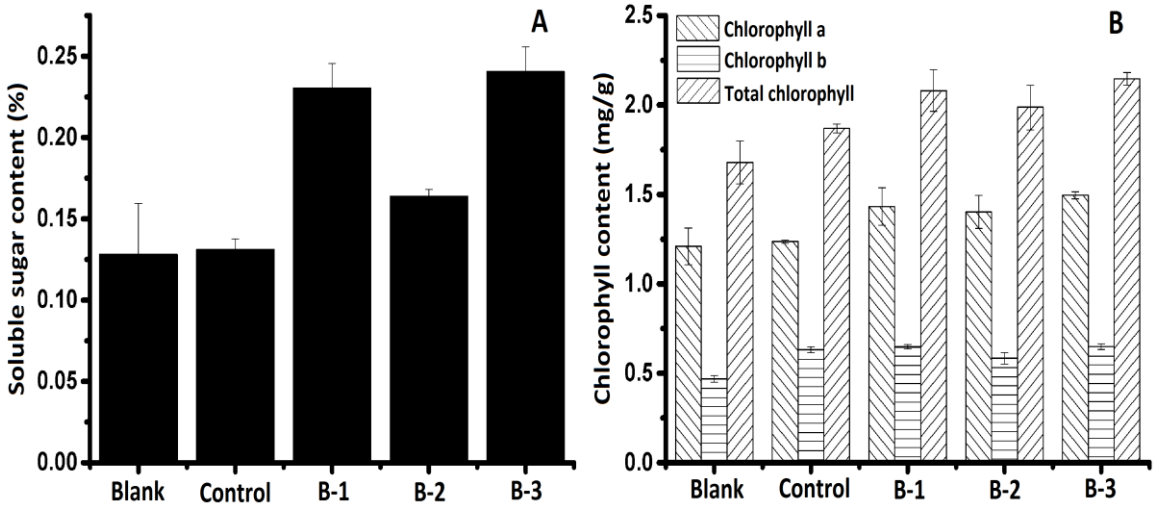

Fig. 5: Soluble sugar (A) and chlorophyll (B) contents of *Glycine max* seedlings.

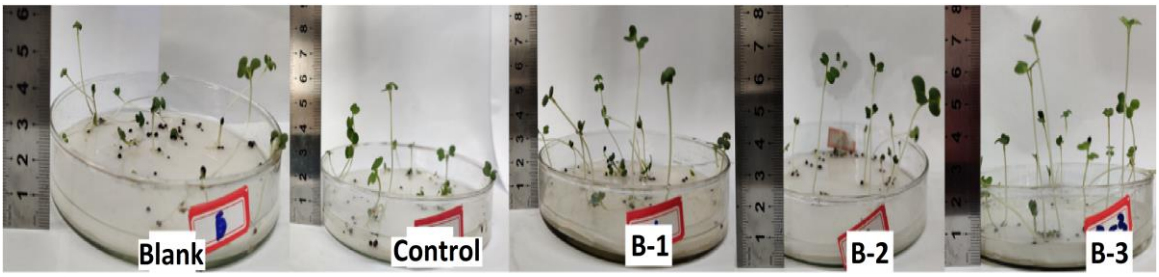

Fig. 6: Photographs of *Brassica napus* after 30 days of culture.

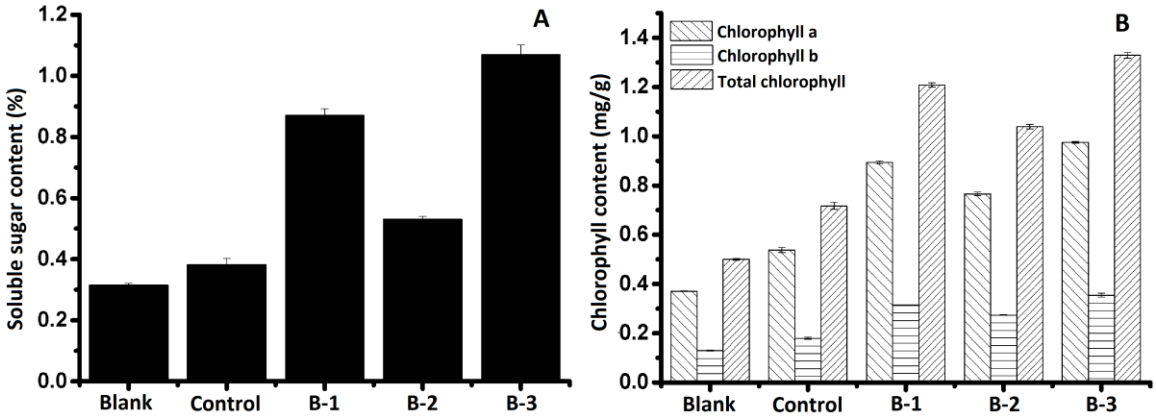

Fig. 7: Soluble sugar (A) and chlorophyll (B) contents of *Brassica napus* seedlings.

Fig. 7A shows the soluble sugar contents in *Brassica napus* seedlings under different breeding conditions. It can be seen that the soluble sugar content of B-3 and B-1 groups was significantly higher than that of other groups, and B-3 was the highest, 1.07%. The sugar content of the B-3 group was 0.75%, 0.68%, 0.20%, and 0.53% higher than that of the blank group, the control group, B-1, and B-2, respectively. These results indicate that the soluble sugar content of *Brassica napus* could be increased with the decrease of molecular weight of collagen hydrolysate.

Fig. 5B provides the chlorophyll a, chlorophyll b, and total chlorophyll contents of *Brassica napus* seedlings. It can be seen that when treated with nutrient solution containing the collagen hydrolysate from limed hides, the chlorophyll content of *Brassica napus* seedlings of the three test groups was significantly higher than that of the control and control groups, among which the chlorophyll content of B-3 group was the highest, with chlorophyll a, b and total chlorophyll contents of 0.97 mg/g, 0.35 mg/g, and 1.32 mg/g, respectively. Compared with the blank group, the increased amount of the chlorophyll a, chlorophyll b and total chlorophyll contents in B-3 group are 0.60 mg/g, 0.22 mg/g and 0.82 mg/g, respectively; compared with the control group, the increase values are 0.4376 mg/g, 0.1743 mg/g and 0.6119 mg/g, respectively; compared with B-1 group, the increase values are 0.0810 mg/g, 0.0402 mg/g and 0.1212 mg/g, respectively; compared with B-2 group, the improvement amounts are 0.2093 mg/g, 0.0805 mg/g and 0.2898 mg/g, respectively. Namely, the order for the total chlorophyll content is B-3 > B-1 > B-2 > control group > blank group. This indicates that compared with the blank and inorganic nitrogen control group, the amino acid groups were more easily absorbed by *Brassica napus* seeds and could promote the generation of chlorophyll. Moreover, the smaller the molecular weight of the collagen hydrolysate was, the higher the chlorophyll content in *Brassica napus* was, indicating that the amino acids with small

molecular weights could promote the absorption by *Brassica napus* and the generation of chlorophyll.

Although the obvious promotion effects were found on the growth of the three crops with different families when three collagen hydrolysates from limed hides were used as nutrient solutions for breeding, there are some differences among the three crops in terms of growth status, soluble sugar, and chlorophyll contents. For example, the germination rates of *Triticum aestivum* and *Glycine max* seeds are greatly higher than that of *Brassica napus* seeds, but compared with the control and blank groups, the germination rate of *Brassica napus* seeds in the tested groups increased more obviously. Similarly, a bigger increase in soluble sugar and chlorophyll contents is observed in the three tested groups containing collagen hydrolysates. These results indicate the promotion effects of collagen hydrolysates extracted from limed hides on the growth of *Triticum aestivum*, *Glycine max*, and *Brassica napus* are different

Conclusion

In summary, we successfully prepared three collagen hydrolysates with different molecular weights from the limed hides by using the enzyme (Alcalase), alkali ($Ca(OH)_2$), and alkali-enzyme extraction procedures, respectively. The three collagen hydrolysates contain many amino acids, but no heavy metals such as Cr, As, Cd, Pb, and Hg, and can be used in the preparation of amino acid fertilizers. Three common crops of different species including *Triticum aestivum*, *Glycine max*, and *Brassica napus* were selected as fertilization objects. Through the determination of their germination rate, plant weight, seedling length, soluble sugar, and chlorophyll contents, the effects of the three collagen hydrolysates with different molecular weights from the limed hides on their growth were investigated, and the conclusions are as follows:

1. Based on the results of the germination rate, plant weight, seedling height, soluble sugar, and chlorophyll contents for the three crops, the order of promotion effect is B-3 > B-1 > B-2 > control group > blank group. In other words, compared with inorganic nitrogen fertilizer, the amino acid fertilizer containing the collagen hydrolysate extracted from the limed hide has a better promotion effect on the growth of three crops of *Triticum aestivum*, *Glycine max*, and *Brassica napus*.
2. The smaller the molecular weight of the collagen hydrolysates extracted from the limed hide is, the more easily they could be absorbed and utilized by the three crops of *Triticum aestivum*, *Glycine max*, and *Brassica napus*, and the better the growth effect was.
3. The promotion effects of the collagen hydrolysates extracted from limed hides on the growth of *Triticum aestivum*, *Glycine max*, and *Brassica napus* are obviously different.

Compared with the inorganic nitrogen fertilizer, the organic fertilizer produced by the collagen hydrolysates extracted from limed hides not only has the characteristic to promote the growth of crops in different families but also is rich in sources [26]. It is a good resource utilization way for limed hide solid waste in the tannery industry.

Supplementary material

Table S1 Composition of limed hide. Fig. S1 The effect of temperature on the Hyp concentration of the resulting EPH. Fig. S2 The effect of time on the Hyp concentration of the resulting EPH. Fig. S3 The effect of Alcalase dosage on the Hyp concentration of the resulting EPH. Table S2 Details of the orthogonal design for enzyme extraction procedure. Table S3 Orthogonal experimental results for enzyme extraction procedure. Fig. S4 The effect of temperature on the Hyp concentration of the resulting APH. Fig. S5 The effect of time on the Hyp concentration of the resulting APH. Fig. S6 The effect of $Ca(OH)_2$ dosage on the Hyp concentration of the resulting APH. Table S4 Details of the orthogonal design for alkali extraction procedure. Table S5 Orthogonal experimental results for alkali extraction procedure. These data can be obtained free of charge at the website of this journal or by contacting the corresponding author of Dr. H. Gu via the email of guhaibinkong@126.com.

Abbreviations

LCHSW: Limed cattle hide solid waste; ECH: enzymatic collagen hydrolysate; ACH: alkali collagen hydrolysate; AECH: alkali-enzymic collagen hydrolysate; ICP-OES: inductively coupled plasma optical emission spectrometer; Hyp: hydroxyproline; GPC: gel permeation chromatography; PEG: polyethylene glycol; B-1: the culture solution prepared by ECH; B-2: the culture solution prepared by ACH; B-3: the culture solution prepared by AECH.

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References

1. X. M. Zhong and X. P. Wu, Present status, existing problems and counter measures of farmland pollution and quality and safety of agricultural products in China, *Chinese Journal of Agricultural Resources and Regional Planning*, **28**, 27 (2007).
2. C. Z. Liu, C. L. Kou and Q. J. Wang, Effects of long-term fertilization on soil fertility and nitrate accumulation and distribution in sand soil, *Chinese Journal of Soil Science*, **27**, 216 (1996).
3. Z. L. Yang, D. L. Wang, M. S. Li and C. S. Li, Strategic thoughts for security of both grain supply and agriculture environment in China, *Chinese Journal of Agricultural Resources and Regional Planning*, **27**, 1 (2006).
4. J. D. Su, Discuss on the harm of excessive application of chemical fertilizer and its countermeasures, *Agriculture and Technology*, **36**, 49 (2016).
5. L. J. Xu, M. L. Liang, J. Q. He and J. L. Chen, Research on Technology and Application of Amino Acid Fertilizer Chelated with Micronutrients, *Guangdong Chemical Industry*, **41**, 38 (2014).
6. C. J. Chu, Application effect of amino acid microelement water-soluble fertilizer in rice field demonstration, *Modern Agricultural Science and Technology*, **1**, 18 (2014).
7. A. Bahari, H. Pirdashti and M. Yaghubi, The effects of amino acid fertilizers spraying on photosynthetic pigments and antioxidant enzymes of wheat (Triticum aestivum L.) under salinity stress, *International Journal of Agronomy & Plant Production*, **4**, 787 (2013).
8. S. Ghasemi, A. H. Khoshgoftarmanesh, M. Afyuni and H. Hadadzadeh, Iron(II)–amino acid chelates alleviate salt-stress induced oxidative damages on tomato grown in nutrient solution culture, *Scientia Horticulturae*, **165**, 91 (2014).
9. Y. Zhang and S. H. Dong, Studies of the Scavenging Capacity of Amino Acids on Active Oxygen Radicals, *Bulletin of Science and Technology*, **7**, 33 (1997).
10. H. J. Wang, L. H. Wu and Q. N. Tao, Advances in the study of biological effect of amino acids, *Chinese Journal of Soil Science*, **34**, 469 (2003).
11. F. Guo, Q. Q. Gao, P. H. Chen, B. R. Duan and Q. J. Wang, Effects of Using Chrome Shavings Organic Fertilizer on Procyanidine in Buckwheat, *Leather and Chemicals*, **34**, 35 (2017).
12. N. Liu, L. F. Gao, Z. W. Ding and Z. P. Cheng, Preparation of amino acid fertilizer with chrome shavings, *China Leather*, **44**, 27 (2015).
13. P. H. Chen, Q. J. Wang, Y. H. Luo and B. R. Duan, Study on Improving the Quality of Buckwheat by Functional Organic Fertilizer Prepared from Chrome Shaving, *Leather Science and Engineering*, **27**, 19 (2017).
14. D. G. Epure, C. F. Cioineag, M. Becheritu, C. Gaidau, E. Stepan and M. Gidea, Use of biofertilizant based on collagen hydrolysate for cereal seed treatment, *AgroLife Scientific Journal*, **7**, 48 (2018).
15. X. C. Cao, L. H. Wu, Q. X. Ma and Q. Y. Jin, Advances in studies of absorption and utilization of amino acids by plants: A review, *Chinese Journal of Applied Ecology*, **26**, 919 (2015).
16. L. Tian, H. Y. Xie, H. Li, X. Luo, Development and Application Effect on Leather Solid Waste and Manure Trial of Amino Acid Liquid Fertilizer, *Hubei Agricultural Sciences*, **54**, 2093 (2015).
17. Y. H. Luo, Q. J. Wang, P. H. Chen and S. Diao, Utilization of waste leather scraps in agriculture, *China Leather*, **46**, 6 (2017).
18. J. Luan, S. Diao, P. H. Chen and Q. J. Wang, The Review of Leather Wastes Recycling, *Leather Science and Engineering*, **26**, 28 (2016).
19. GB5009.3−2016, Determination of water content in foods, 2016.
20. GB5009.4−2016, Determination of ash content in foods, 2016.
21. GB5009.5−2016, Determination of protein in foods, 2016.
22. GB5009.6−2016, Determination of fat in foods, 2016.
23. Y. L. Chi, Z. H. Wen, M. Cui, X. P. Liao, B. Shi, Determination of collagen hydrolytic degree, *China Leather*, **39**, 13 (2010).
24. P. Wang, J. J. Zhang, M. M. Lv, Z. H. Ma, Y. Chen, S. Z. Lin, Expression analysis of NIPAs gene in different phenotypes of Chinese fir (Cunninghamia lanceolata), *Journal of Fujian*

Agriculture and Forestry University (Natural Science Edition), **48**, 325 (2019).

25. M. X. Zhang, X. S. Kong, X. P. Guo, L. Q. Li, Study on the colorimetric condition of soluble sugar by anthrone method, *Journal of Luoyang Agricultural College*, **17**, 24(1997).
26. A. Gul, B. Kunwar, M. Mazhar, S. Faizi, D. Ahmed, M. R. Shah, S. U. Simjee, Rutin and rutin-conjugated gold nanoparticles ameliorate collagen-induced arthritis in rats through inhibition of NF-kappa B and iNOS activation, *International Immunopharmacology*, **59**, 310 (2018).