Study on Extraction Technology and Functional Activity of Sika Deer Velvet(Residue) Collagen

Shiru Bo[1, a]，Xiaolin Zhang [2, b]，Quankai Wang [3, c]

[1]Jilin agricultural science and technology college Jilin 132101

[2]Jilin agricultural science and technology college Jilin 132101

[3]Jilin agricultural science and technology college Jilin 130000

[a]email, [b]email, [c]email,

Keyword：Sika Deer Velvet Antler, Collagen, Functional Activity

Abstract: **Objective**:Extraction method and functional activity of collagen that is extracted from residue that is insoluble in any medium polar solvent by ultrasonic extraction andsupercritical CO_2 extraction are studied. Finally the optimal enzymolysis conditionsare obtained.**Methods**: Quantitative content of collagen in deer antler (residue) by quantitate hydroxyproline. Sika deer antler collagen is prepared by enzymolysis of papain. According to single factor test and the orthogonal design, the optimal enzymatic conditions aredetermined by the extraction rate of collagen as evaluation indicator. And functional activity are tested which include antioxidant capacity, hydroxyl free radical scavenging ability, DPPH scavenging ability of the test and keratinocyte cell test.**Results**: The content of collagen in the deer antler (residue) is 4.5795%,the best extraction process is 2% optimum enzyme dosage , PH 6.0, 1:20 solid-liquid ratio, enzymolysis time 66h at 10℃. The extraction rate of collagen of the deer antler is 54.59%.The protein is collagen by UV spectroscopy.Functional tests are showed a good functionality and growth and reproduction capacity.

Introduction

The velvet that is nor ossified pilose antler of sika deer or red deer has unique biological activity and chemical composition. The traditional product processing of getting the deer velvet is backward,which lead to low effective active ingredient and utilization. Collagen physiological function that is lose weight, blood pressure and blood fat, immune regulation, antioxidant and so on is widely used in medicine, food, cosmetics and other fields. Currently collagen of raw materials are mostly animal skin, bone, waste leather in which there are more Heavy metals, pesticides and other harmful substances on the market.Because of velvet growth cycle less than six months,there are less harmful substances. In this paper, extraction method and functional activity of collagen that is extracted from residue that is insoluble in any medium polar solvent by ultrasonic extraction and supercritical CO_2 extraction are studied .

Experimental Materials

Main Raw Materials and Reagents. Residue that is insoluble in any medium polar solvent(Yitong Ji Yun deer industry), perchloric acid, isopropyl alcohol, n-propanol, chloramine T, stannous chloride, L-hydroxyproline standard, bovine serum albumin, papain(80 million U /g), $FeSO_4$,H_2O_2 ,DPPH (Changchun aoke Biological Technology Ltd), Other reagents are of analytical grade.

Main Equipment. GB204 electronic analytical balance(SARTORIUS Ltd)，PH meter(Shanghai

huyue Science and Technology Instrument Ltd), W201B constant temperature water bath(Shanghai Shensheng Technology Ltd), UV2800 UV Visible Spectrophotometer(Shimadzu), CHRiST vacuum freeze dryer(CHRiST), High speed refrigerated centrifuge(Sigma), Dialysis bag(Changchun aokeBiological Technology Ltd), CO_2 incubator(Shanghai hecheng equipment Manufacturing Ltd)

Experimental Method

Determination of Collagen Content. L(-)-hydroxyproline standard curve is established according to GB9695.23-90 “Content determination of L-hydroxyproline of meat and meat products”. According to the determination of hydroxyproline, hydroxyproline is released When the velvet are hydrolyzed by stannous chloride. Absorbance and the content of hydroxyproline in sika deer velvet are tested. Quantitative calculation of the content of collagen in the sika deer velvet through the terrestrial animals commonly used 7.1 as a conversion factor [2].

Pickling. According to pre-experiment and single factor test,the optimum conditions for decalcification of sika deer velcet are 4% HCL, 1:15solid-liquid ratio, 48h pickling time at 4°C. The decalcification rate can reach 96.42% In this condition.

Impurity Removal. In this experiment, NaCl solution is selected as the main reagent for the dephosphorization protein. According to pre-experiment and single factor test,the optimum conditions for protein removal of sika deer velcet are 4% HCL, 1:10 solid-liquid ratio, 12h removal time.

Extraction with Papain and Acid. In this experiment, papain and citric acid are combined to extract to Keep alive and low antigenic at low temperature. The enzymolysis liquid has been rancid at 25°C through pre-experimentation .So the temperature of enzymatic extraction is 10°C.

According to pre-experiment and single factor test, the factors and levels of orthogonal test are determined. Through the orthogonal design scheme of $L_9(3^4)$, screen the optimum match of enzyme dosagel, PH, solid-liquid ratio and enzymatic time by the extraction rate of collagen and other proteins content as evaluation indicator.

Preparation of standard curve of other proteins: 50 mg bovine serum albumin at concentration of 0mg/ml、0.2mg/ml、0.3mg/ml、0.6mg/ml、0.9mg/ml、1.2mg/mldetermine absorbance at wave length of 280nm.The standard curve with the absorbance A and the content of bovine serum albumin is the ordinate and abscissa.The standard curve equation:$y = 0.5844x - 0.036$，$R^2 = 0.9985$.

Tab 1 Levels of factors for extract collagen by papain with citric acid

factor / Level	(A)Enzyme dosage（%）	(B)PH value	(C)Volume ratio	(D)time（h）
1	1.0%	5	1:12	66
2	2.0%	6	1:15	72
3	3.0%	7	1:20	78

Purification and Detection of Crude Protein. All the collagen is salted under neutral conditions,when the concentration of sodium chloride is 4.0mol/L or 20%[4].Nacl is slowly added the sika deer antler collagen enzymatic solution . Fully stir and static 10-24h, 9000 rpm speed centrifugation. The precipitate is collected and dissolved in 0.5 mol / L acetic acid. Dialyse by 0.5 mol / L acetic acid in order to remove the salt from the precipitate. The concentration of non-collagen decreased to below 5% and vacuum freeze.

The material by dialysing is dissolved with 0.5 mol / L glacial acetic acid and diluted with distilled water.The purity of velvet collagen is tested at wave length of 280nm by UV Visible Spectrophotometer[5].

Functional Determination. Determination of free radical activity of superoxide anion by pyrogallol automatic oxidation [6].

Determination of self-oxidation rate of blank samples: 0.1mL constant temperature of 45mmol /L pyrogallol solution is added to mixed solution that are 5 mL Tris-HCl buffer with pH 8.20 and 0.5 ml water which is constant to 10min at 25℃.Absorbance is tested at wave length of 280nm by UV Visible Spectrophotometer every 30s and continue ten times and self-oxidation rate V_1 is calculated.

Determination of self-oxidation rate of samples: 0.1mL constant temperature of 45mmol /L pyrogallol solution is added to mixed solution that are 5 mL Tris-HCl buffer with pH 8.20 and 0.5 ml 10mg/mL sample solution which is constant to 10min at 25℃.Absorbance is tested at wave length of 280nm by UV Visible Spectrophotometer every 30s and continue ten times and self-oxidation rate V_1 is calculated[7].

The inhibition rate is calculated from the autoxidation rate of pyrogallol, The formula is as follows: Inhibition rate=（V_1-V_0）/V_1×100%,(V_0 and V_1 are the autoxidation rate of pyrogallol of blank and sample groups).

According to table 2,the absorbance is calculated at wavelength of 510 nm by spectrophotometry [8].

Tab 2 Sample test of OH^- scavenging ability

Absorbance	Solution composition
A_0	1mL 9mmol/L salicylic acid-Ethanol Solution＋1mL 9mmol/L $FeSO_4$＋1mL Distilled water＋1mL 8.8mmol/L H_2O_2
A_1	1mL 9mmol/L Salicylic acid-ethanol solution＋1mL 9mmol/L $FeSO_4$＋1mL10mg/mL Sample solution＋1mL 8.8mmol/L H_2O_2
A_2	1mL 9mmol/L Salicylic acid - ethanol solution＋1mL 9mmol/L $FeSO_4$＋1mL distilled water＋1mL10mg/mL Sample solution

The removal rateiscalculatedas:clearance rate=$\frac{A_0-(A_1-A_2)}{A_0}$X100%

A_0 indicates the absorbance of the blank control solution

A_1 indicates the absorbance of the solution to be measured

A_2 indicates the absorbance of the liquid background of the sample to be measured without the developer

(1) Mixed solution that are 2mL DPPH solution and 2mL 70% ethanol solution measured at wave length of 516nm by 70% ethanol as a reference and A_1 isrecorded.

(2) Mixed solution that are 2mL 70% of ethanol solution and 2mL 10mg/mL of the sample solution measured at wave length of 516nm by 70% ethanol as a substance and A_2 is recorded.

(3) Mixed solution that are 2mL 10mg /mL of the sample solution, 2mL DPPH solution and 2mL 70% ethanol solution measured at wave length of 516nm by 70% ethanol as a substance and A_3 is recorded.

Calculation formula of the removal rate=$\frac{1-(A_3-A_2)}{A_1}$×100%Biological activity and Sample different concentrations is tested which are antioxidant capacity, hydroxyl scavenging ability and DPPH scavenging ability.

Keratinocytes Test. Keratinocytes that are collected after processing are cultivated of 2-3 days at 37°C in 5% CO_2incubator.Aftercells conically multiply, the velvet collagen with the same concentration is cultivated which is coated on a 35ml culture dish as a substrate. After 2hours, That cell density of Keratinocytes reached $4\times10^4/cm^2$ are add and cultivated of 10hours. Bioactivity of

pilose antlercollagen is tested by microscope. Cell growth is an evaluation index[10].

Results and Analysis

Determination of Collagen Content. The linear relationship between absorbance and L(-)-hydroxyproline concentration is: y=0.2322x+0.0012(R^2=0.9998).

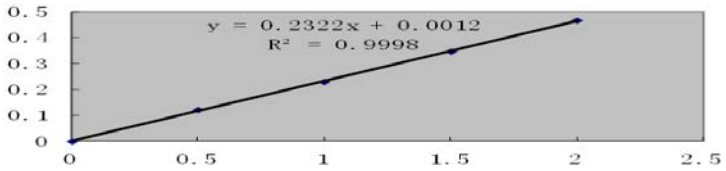


Fig.1 The standard curve of L(-)-hydroxyProlin

According to the formula: $C=\frac{X\times 250\times \frac{200}{V_1}\times 10^{16}}{m}\times 100=\frac{5X}{mV_1}$,the content of L (-)- hydroxyproline in the deer antler is 0.645%.The content of collagen in the deer antler Is 4.5795%. Under the experimental conditions by the conversion factor 7.1.

C on behalf of the content of L (-) - hydroxyproline in the deer antler (%)

X on behalf of Check the corresponding L (-) - hydroxyproline from the standard curve(ug/mL）

m on behalf of weigh the quality of the deer velvet（g）

V_1 on behalf of the volume of the filtrate is drawn from 200 mL volumetric flask,(mL)

Determination of Optimum Enzymatic Hydrolysis Conditions of Papain. According to single factor test, the factors and levels of orthogonal test are determined. Through the orthogonal design scheme of $L_9(3^4)$, screen the optimum match of enzyme dosagel, PH, solid-liquid ratioand enzymatic time.

Tab 3 The results of orthogonal experiment

Processingnumber	Enzymedosage	PH	Volumeratio	time	Collagen(mg)	Protein(mg)
1	1	1	1	1	135.14	28.82
2	1	2	2	2	138.71	29.09
3	1	3	3	3	139.07	28.68
4	2	1	2	3	146.96	29.86
5	2	2	3	1	148.19	30.66
6	2	3	1	2	148.13	30.47
7	3	1	3	2	149.69	32.07
8	3	2	2	3	141.13	29.26
9	3	3	1	1	140.89	29.21
K1	151.25	158.16	159.92	159.42		
K2	162.74	160.42	159.11	159.90		
K3	165.46	160.87	159.42	159.13		
k_1	50.42	52.32	53.04	53.14		
k_2	54.25	52.72	53.30	53.80		
k_3	55.15	53.79	54.47	53.38		
R	4.74	0.89	0.43	0.24		

Tab 4 Anova table

The source of cariance	square of deviance	Degrees of freedom	variance	F value	Significance
A	37.93	2	18.97	446.35	P＜0.01
B	1.39	2	0.70	16.38	P＜0.1
C	0.28	2	0.14	3.34	P＞0.1
D	0.085	2	0.043	1.00	P＞0.1

$F_{0.1(2,2)}$=9.00 $F_{0.05(2,2)}$=19.00 $F_{0.01(2,2)}$=99

Based on the orthogonal test results ,the order of influence is A＞B＞C＞D that enzyme dosagel>PH>solid-liquid ratio>enzymatic time . The results of variance analysis show that the enzyme dosagelhavet effect on experimental results. PH is statistical significance. From the point of view of the protein, enzyme dosagel is significant statistical significance. The best combination of factors is $A_2B_2C_3D_1$ in which the dosage of 2% enzyme dosagel, PH 6.0,1:20 solid-liquid ratio, 66h enzymatic time. The extraction rate of collagen is 56.59%.

Sika Deer Antler Collagen UV Analysis Results.

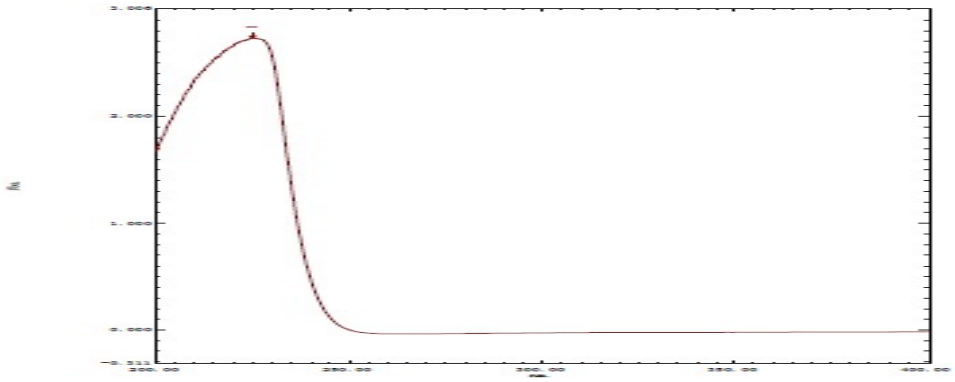


Fig.2 Sika Deer Velvet collagen UV scan

The test results are shown the protein is high purity collagen,because there is a strong absorption peak at 225 nm and a very low peak at 250-290 nm.

Functional Determination. Sika deer antler collagen solution is respectively diluted to four different concentrations that are 10mg/mL、20mg/mL、30mg/mL、 40mg/mL and test absorbance (A_1、A_2、A_3、A_4) .Physiological functions are detected which are antioxidant capacity, hydroxyl free radical scavenging ability and DPPH scavenging ability by 0.05mg/mL VC as control and compare the scavenging of different concentrations of the sample itself.

Tab 5 the scavenging results of Sika Deer Velvet collagen on the O^{2-}

species	A_1	A_2	A_3	A_4	VC
clearance rate（%）	25.96	27.42	28.63	28.71	38.83

Tab 6 the scavenging results of Sika Deer Velvet collagen on the OH^-

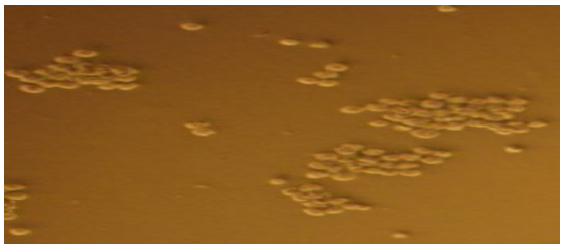
species	A_1	A_2	A_3	A_4	VC
clearance rate（%）	30.21	35.53	38.64	40.07	88.61

Table 7 the scavenging results of Sika Deer Velvet collagen on the DPPH

species	A_1	A_2	A_3	A_4	VC
clearance rate（%）	29.06	40.27	44.39	45.61	88.61

Physiological functional tests are showed there are a good functionality.With the increase in the concentration of collagen deer antler collagen, physiological functions that are the scavenging ability of O^{2-}, OH^- and DPPH radicals gradually grow.

Keratinocytes Test



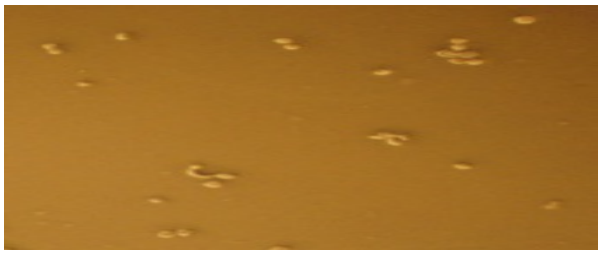


Fig.3 Culture of keratinocytes on spermatid collagenFig.4 Petri dish

The figs3, 4 are showed that the cells are cultured on the deer antler collagen have good growth and reproduction ability. The collagen which Biological activity of is not lost has reproduction capacity.

Conclusion

Through the optimization of the extraction process for the deer antler (residue),better purity of collagen is obtained. The collagen has the role of promoting cell regeneration in accordance with functional research.

The research still need further study and discussion.For instance：（1）Increase the use of specific products in order to large-scale production of sika deer antler collagen to product.(2)Find the characteristic active ingredient of the deer antler,make sure a characteristic activity factor and extract.(3)Strengthen the pharmacological research of the sika deer antler,detection of each pharmacological effect whichare produced by which componentt of the velvet.

Acknowledgements

Fund Project: Project:Jilin Agricultural Science and Technology College Youth Fund Project (11901-2015002)

References:

[1]WangJiazheng, Fan Ming. Protein Technology Handbook [M] Science Press, 2000.8.

[2]GB 9695.23-90 "Determination of L (-) - hydroxyproline in meat and meat products"

[3]Yongjing,Yu.FujimotoMitsuji(ed.),Liu Ping(translation).Science and Technology of Collagen [M].Shanghai College of Traditional Chinese Medicine, 1992.

[4]JiangTingda,Collagen and Collagen [M]. Chemical Industry Press, 2006.2.

[5]Tao Wei Sun,LiWei&JiangYongming, etc. Protein Molecular Basis [M] Higher Education Press, 1995.10: 251.

[6]GuoXuefeng, Yue Yongde&Tang Feng, et al.Evaluation of antioxidant capacity of bamboo leaf

extract by clearing superoxide anion free radical method[J].SpectroscopyandSpectral Analysis,2008,28 (8): 1823-1826.

[7]MIAOZhi-wei, ZHOU Wei-hong&LIU Xiao-lan, et al. Synthesis,characterization and activity determination of superoxide dismutase model compounds[J].Chinese Journal of Organic Chemistry, 1999,19,537-541.

[8]Gordon,M.H. The meachnism of antioxidant action in vitro[J].Food antioxida-nts,1990,1-18

[9]DENG Sheng-guo, DENG Ze-yuan&HUANGLi.Study on Antioxidant Activity of Flavonoids in Lotus Leaves [J].Food Science and Technology 2006.7,274-276.

[10]StuartZhenqiang, Wu Junzheng, editor. Cell culture [M]. Xi'an: World Book Publishing Company, 1996, 69-83.