Electronic Supplementary Information

Optimization of *Jiuzao* protein hydrolysis conditions and antioxidant activity *in vivo* of *Jiuzao* tetrapeptide Asp-Arg-Glu-Leu by elevating Nrf2/Keap1-p38/PI3K-MafK signaling pathway

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2. Materials and methods

Relate with 2.1-Materials used in the experiment

PrimeScript™ RT reagent Kit with gDNA Eraser, SYBR® Premix Ex Taq™ II (Tli RNaseH Plus) and DL2,000 DNA Marker were obtained from TaKaRa Co., Ltd. (Beijing, P.R. China). Bovine serum albumin (BSA) kit was obtained from Sigma-Aldrich, (St. Louis, USA). The human interleukin 1β (IL-1β), interleukin 6 (IL-6), tumor necrosis factor-α (TNF-α), and nitric oxide (NO) ELISA kit were purchased from AB clonal Biotechnology Co., Ltd. (Wuhan, Hubei, P.R. China). Oral fluids were purchased from Solarbio Co., Ltd (Beijing, P.R. China). Simulated gastric fluids and small intestinal fluids were obtained from Leagene Co., Ltd (Beijing, P.R. China). Antibody against Nrf2 (MAB3925), antibody against Keap1 (MAB3024), antibody against SOD1 (AF3787), anti-CAT antibody (AF3398) and anti-GPX1 Antibody (AF3798) were obtained from R&D Systems (Minnesota, USA). Rabbit anti-p-p38 antibody (4511), rabbit anti-p38 antiobody (6960), rabbit anti-p-PI3K antibody (182651), and rabbit anti-PI3K antibody (151549) were obtained from Cell Signaling Technology (Boston, USA). Rabbit anti-MafK antibody (orb580107) were obtained from Biorbyt (Cambridge, UK). HRP-conjugated Affinipure Goat Anti-Mouse IgG (SA00001-1), HRP-conjugated Affinipure Rabbit Anti-Goat IgG (SA00001-4), and HRP-conjugated Affinipure Goat Anti-Rabbit IgG (SA00001-2) secondary antibody were obtained from ProtenTech (Illinois, USA).

Relate with 2.4.1-Sample collections

Tissue homogenate: Liver, heart, and kidney were excised immediately after sacrifice, then rinsed in 0.9% cold physiological saline, vacuum packed. Liver, heart, and kidney tissue samples were rinsed with ice brine. Then they were put into the tubes and cut into small ones. Small pieces of tissues were then fully homogenized on ice with an electric homogenizer. The homogenized liquid was transferred into clean centrifuge tubes and added with ice saline (w:v=1:9). The tissue homogenate was centrifuged at 2000 × *g* for 10 min. Subsequently, the supernatant was gently absorbed and moved to new tubes for further use.

Serum: The blood was collected from aorta abdominals then the serum was separated by centrifugation at 4000 × *g* for 10 min at -4℃.

Preparation of total, nuclear and cytoplasmic proteins for western blot: For total protein, the tissue blocks were chopped and placed in centrifuge tubes. They were mixed with RIPA cell lysate pre-protease inhibitor (w:v =1:5) to fully homogenize and placed on ice for 30 min to further fully crack. The lysate was centrifuged at 12000 × *g* for 10 min. Finally, the supernatant was transferred to the new tubes, placed on ice, and prepared for protein concentration determination.

For nuclear and cytoplasmic proteins, the tissues were cut into small piece. Then the nuclear and cytoplasmic protein was extracted with the manufacturer's protocol of nuclear extraction kit.

Related with 2.4.5-The sequences of the primers

Nrf2: 5'- AAGATGCCTTGTACTTTGAAGACTGT-3' and 5'-GGAAAATAGCTCCTGCCAAACTT-3'; Keap1: 5'-

TGCTCAACCGCTTGCTGTAT-3’ and 5’- TGGTATTCATTGGTGTAATCATCC-3’; MafK: 5’-CCCAAGCCCAACAAGACATT-3’ and 5’-CTCCAGCTCCTCCTTCTGTG-3’; p38: 5’-TGAAGCACATGAAGCACGAG-3’ and 5’-GAACGTGGTCATCGGTAAGC-3’; PI3K: 5’-TCCTGAACTGGCTCAAGGAG-3’ and 5’-TCTATGTGGAAGAGCTGGCC-3’; CAT: 5’- CCTTTTTGCTTACCCAGACACT-3’ and 5’-GGTAGGGACAGTTCACAGGTATC-3’; GSH-Px1: 5’-GGAGAATGGCAAGAATGAAGA-3’ and 5’- GGAAGGTAAAGAGCGGGTG-3’; SOD1: 5’-GCAAAGGTGGAAATGAAGAAAG-3’ and 5’-CCAATCACACCACAAGCCA-3’; HO-1: 5’- AGCACAGGGTGACAGAAGAG-3’ and 5’- AACTCTGTCTGTGAGGGACT-3’; GAPDH: 5’-CCTTCCGTGTTCCTACCCC-3’ and 5’- GCCCAGGATGCCCTTTAGTG-3’.

The cycle conditions of real-time PCR

95°C for 30 s; 40 PCR cycles (95°C,5 s; 60°C for 40 s) (collect fluorescence). In order to establish the melting curve of the PCR product, after the amplification reaction, the condition turned to 95°C for 10 s; 60°C for 60 s; 95°C for 15 s.

3. Results

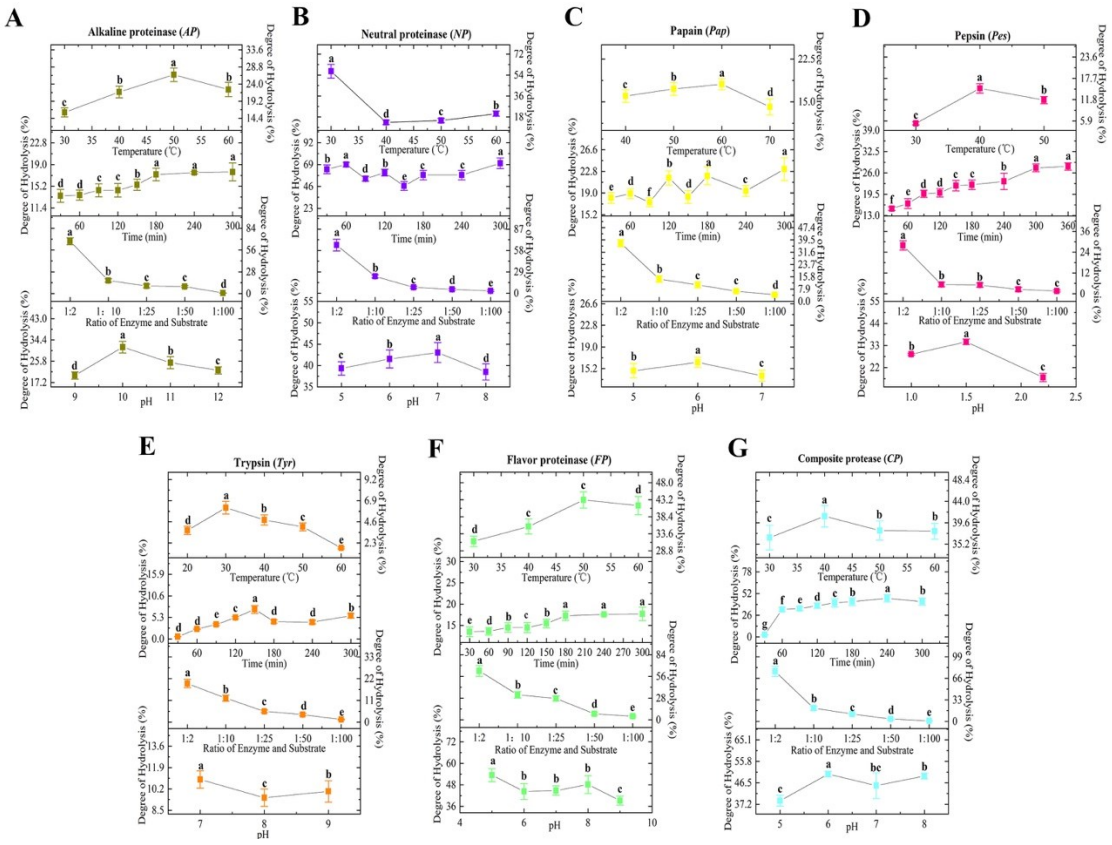


Fig. S1. Optimization conditions of seven proteinases in *Jiuzao* protein hydrolysis. Different letters indicate significant difference at $p < 0.05$ among different groups.

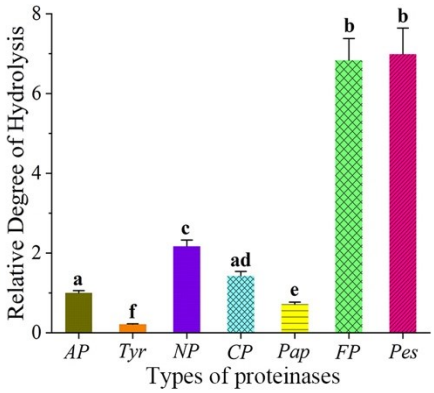
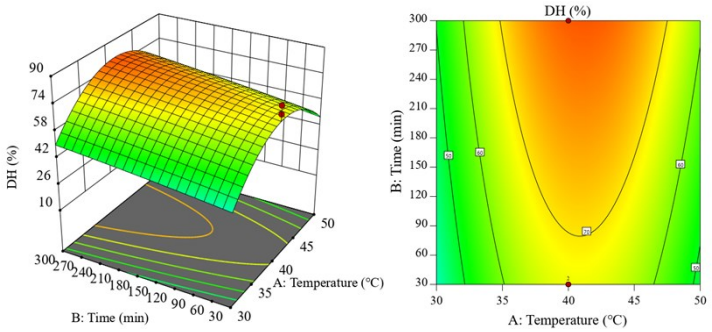
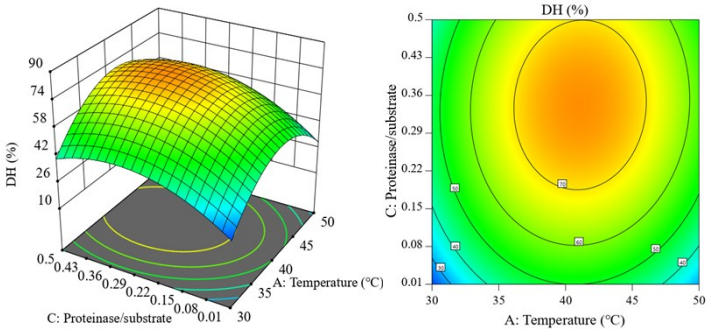


Fig. S2. DHs of proteinases under optimum hydrolysis conditions by one-single factor experiment. Different letters indicate significant difference at $p < 0.05$ among different groups.

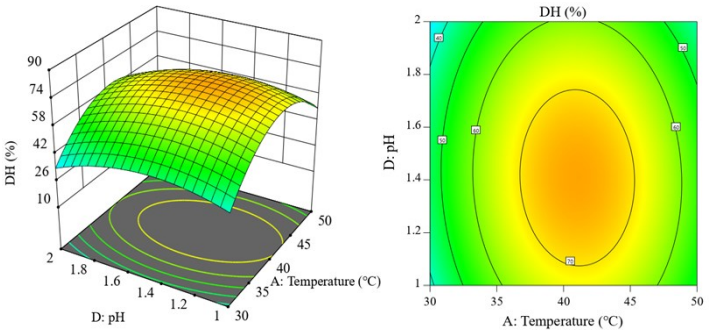
A


DH (%)
90
74
58
42
26
10
300
270
240
210
180
150
120
90
60
30
B: Time (min)
30
35
40
45
50
A: Temperature (°C)
DH (%)
B: Time (min)
A: Temperature (°C)

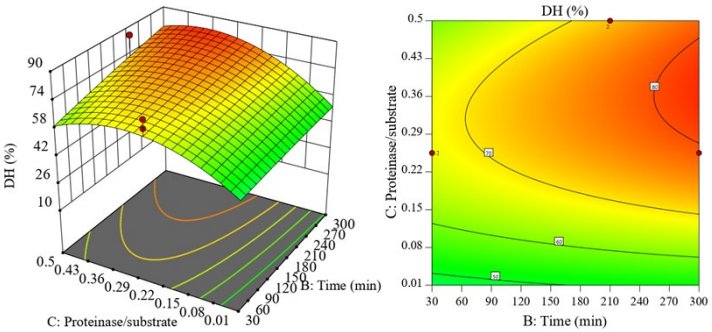
B


DH (%)
90
74
58
42
26
10
0.5
0.43
0.36
0.29
0.22
0.15
0.08
0.01
C: Proteinase/substrate
30
35
40
45
50
A: Temperature (°C)
DH (%)
C: Proteinase/substrate
A: Temperature (°C)

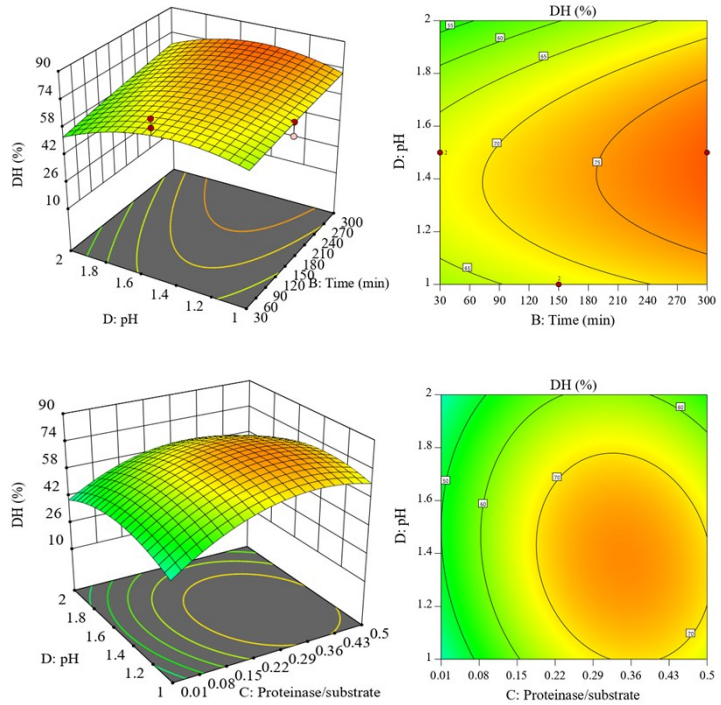
C


DH (%)
90
74
58
42
26
10
2
1.8
1.6
1.4
1.2
1
D: pH
30
35
40
45
50
A: Temperature (°C)
DH (%)
D: pH
A: Temperature (°C)

D


DH (%)
90
74
58
42
26
10
0.5
0.43
0.36
0.29
0.22
0.15
0.08
0.01
C: Proteinase/substrate
30
60
90
120
150
180
210
240
270
300
B: Time (min)
DH (%)
C: Proteinase/substrate
B: Time (min)

E



F

Fig. S3. The response surface and contour charts among different factors. (A) Temperature with time; (B) Temperature with proteinase/substrate; (C) Temperature with pH; (D) Time with proteinase/substrate; (E) Time with pH; (F) Proteinase/substrate with pH.

Fig. S4. Interaction effect of different proteinases on DHs. Different letters indicate significant difference at $p < 0.05$ among actual value of DH; *** $p < 0.001$, ** $p < 0.01$, and * $p < 0.05$ means significant different between theoretical DH of different combinations vs actual values of DH.

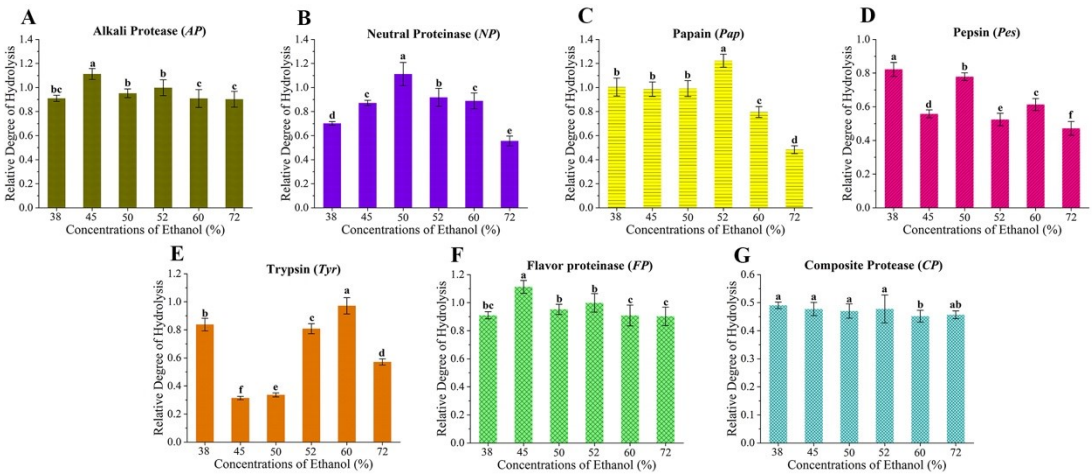


Fig. S5. Effect of different concentrations of ethanol on DH of *Jiuzao* protein hydrolysis. Different letters indicate significant difference at $p < 0.05$ among different groups.

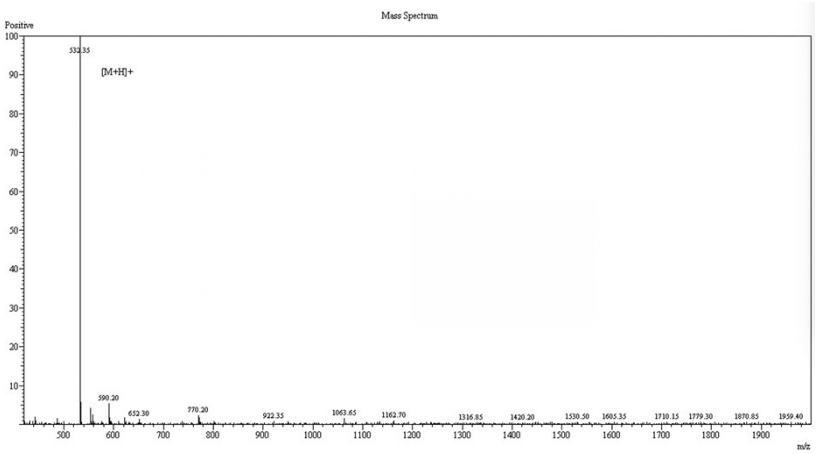


Fig. S6. MS/MS spectrograms of synthetic peptide DREL.

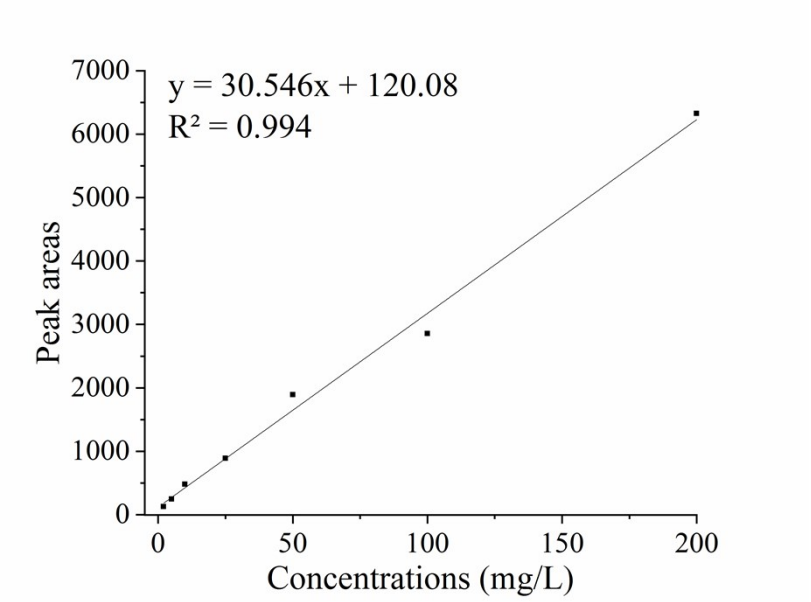


Fig. S7. Standard curve of DREL for quantification

Table S1. Table of test factor levels.

Independent variable	Letters	Levels									
Temperature (℃)	A					30	40	50			
Time (min)	B	30	60	90	120	150	180	210	240	270	300
Proteinase/substrate	C				0.01	0.1325	0.255	0.3775	0.5		
pH	D					1	1.5	2			

Table S2. The results of RSMA Optimal (Custom) Design.

Runs	Factors				DH (%)
	A	B	C	D	
1	50	180	0.1325	1.5	27.68
2	40	210	0.1325	2	68.56
3	40	300	0.01	1	35.36
4	30	90	0.3775	2	24.04
5	30	90	0.3775	2	23.07
6	30	30	0.5	1	29.92
7	40	150	0.255	1	59.46
8	40	30	0.255	1.5	76.54
9	40	210	0.5	1.5	84.98
10	40	30	0.255	1.5	71.5
11	50	30	0.5	2	25.94
12	30	300	0.01	2	18.85
13	50	270	0.1325	1	55.5
14	30	300	0.3775	1	46.35
15	40	300	0.255	1.5	64.58
16	40	210	0.5	1.5	67.51
17	30	240	0.1325	1.5	46.32
18	30	90	0.01	1.5	20.84

19	50	30	0.5	1	34.19
20	50	180	0.1325	1.5	54
21	50	30	0.01	1	21.07
22	50	300	0.5	1	60.04
23	50	300	0.3775	2	48.32
24	40	150	0.255	1	67.512
25	40	60	0.01	2	30.65

Table S3. The response surface Optimal (Custom) experiment with regression model variance analysis.

Run	**Sum of Squares**	**Degree of Freedom**	**Mean Square**	**F-value**	**p-Value**	**Significance**
Model	8349.40	14	596.39	3.85	0.0189	Significant
A-Temperature	256.51	1	256.51	1.66	0.2271	
B-Time	431.61	1	431.61	2.79	0.1260	
C-Proteinase/substrate	1040.59	1	1040.59	6.72	0.0269	
D-pH	151.57	1	151.57	0.9786	0.3459	
AB	5.47	1	5.47	0.0353	0.8547	
AC	11.95	1	11.95	0.0772	0.7868	
AD	11.02	1	11.02	0.0712	0.7951	
BC	85.92	1	85.92	0.5547	0.4735	

BD	16.50	1	16.50	0.1065	0.7509	
CD	62.82	1	62.82	0.4056	0.5385	
A^2	2820.58	1	2820.58	18.21	0.0016	
B^2	0.8874	1	0.8874	0.0057	0.9412	
C^2	683.05	1	683.05	4.41	0.0621	
D^2	424.99	1	424.99	2.74	0.1286	
Residual	1548.79	10	154.88			
Lack of Fit	1004.23	5	200.85	1.84	0.2590	Not significant
Pure Error	544.56	5	108.91			
Cor Total	9898.19	24				

Table S4. Damage grades summary of H&E in liver, kidney, and heart.

Organs	Groups	Damage grades					Total
		++++	+++	++	+	-	
		Number of rats					
Liver	Control			1	2	7	10
	AAPH	4	4	2			10
	VC				5	5	10
	Silymarin				6	4	10
	DREL-H				4	6	10
	DREL-M		1	3	4	2	10
	DREL-L	2	3	4	1		10
Kidney	Control			2	2	6	10
	AAPH	3	4	2	1		10
	VC			1	3	6	10
	Silymarin		1	1	5	3	10
	DREL-H			2	4	4	10
	DREL-M		1	3	4	2	10
	DREL-L	1	3	4	2		10
Heart	Control				1	9	10
	AAPH	1	2	2	3	2	10
	VC			1	2	7	10
	Silymarin			3	1	6	10
	DREL-H				2	8	10
	DREL-M		1	2	3	4	10
	DREL-L		1	3	2	4	10

Where ++++ means the inflammation is widely distributed and multiple inflammatory foci or nodules could be seen in one visual field; +++ means there are many inflammatory foci or small necrosis could be seen in almost every visual field; ++ means fewer inflammatory foci, one inflammatory foci could be seen in multiple visual field; + means few inflammatory foci, or inflammation, or swelling of cells; - means no inflammation, bleeding, and swelling.