Technological Optimization for Alcalase Hydrolysis of Ryegrass Leaf Protein Concentrate (LPC)

LIU Yan, XUE Zheng-lian, WEI Ming
(Department of Biochemistry Engineering, Anhui University of Technology and Science, Wuhu 241000, China)

Abstract: The effects of pH, temperature and proportion of enzyme to substrate (E/S) on the hydrolysis of ryegrass leaf protein concentrate (LPC) with alcalase were investigated by single factors and $L_9(3^4)$ orthogonal test. The results indicated that the optimal pH, temperature and E/S for hydrolysis of ryegrass LPC were 10.5, 45 ℃ and 1750 units per gram respectively. The protein hydrolysate analysis was performed by high performance liquid chromatography (HPLC). There were 7 kinds of peptides. Their relative molecular weights are between 102 and 14502 and most of them are 249, accounted for 21.13%. Amino acids analysis was performed by automatic amino acid analyzer (AAAA). The total content of free amino acids in hydrolysates accounted for 3.233%. The proportion of essential amino acids to total free amino acids is 30.776%, while the proportion of sapor amino acids to total free amino acids is 38.324%, and the proportion of drug-effective amino acids to total free amino acids is 75.286%.

Key words: optimal conditions; ryegrass LPC; the component of amino acid

碱性蛋白酶对黑麦草叶蛋白水解作用的研究

刘 艳，薛正莲，魏 明
(安徽工程科技学院生化工程系，安徽 芜湖 241000)

摘 要： 通过单因素和$L_9(3^4)$正交试验，系统研究了碱性蛋白酶水解黑麦草叶蛋白的水解最佳工艺。确定出该酶水解黑麦草叶蛋白的最佳工艺参数是温度为45℃，pH10.5，E/S=1750U/g，反应时间为10h。利用高效液相色谱和氨基酸自动分析仪分析得出最佳工艺条件下的水解液中共有7个部分的肽段，其相对分子质量分布在102～14502之间，其中数均相对分子质量249的肽含量最多，约占21.13%。氨基酸自动分析仪图谱分析得出最佳工艺条件下的酶解液中总游离氨基酸的含量为3.233%，其中必需氨基酸占总氨基酸的30.776%，鲜味氨基酸占38.324%，药效氨基酸占75.286%。

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作者简介：刘艳(1981-)，女，硕士研究生，研究方向为酶工程。

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关键词：最佳条件；黑麦草叶蛋白；氨基酸组成
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1 Introduction

Ryegrass, one of the most important pastures cultivated in the world, is of increasing interest as a source of available proteins. LPC(leaf protein concentrate) can be obtained from fresh ryegrass[1]. The contents of Ca, P, Mg, Fe, Zn in LPC are very high, about 5～8 times higher than the seed of several plants. The contents of carotene and lutein in LPC are respectively 20～30 times and 4～5 times higher than the other leaves. LPC has no cholesterol, but many physiological functions such as health and physical fitness improvement. It is considered as a high-quality food by FAO, and also a new type of high-value protein resources in the future[2-4].

Protein can be modified by enzymatic hydrolysis, yet altering, for instance, solubility, emulsion and foam properties[5]. It has been reported that protein hydrolysates could be served as suitable sources of protein for human nutrition because of their gastrointestinal absorption, especially dipeptides and tripeptides which seem to be more effective than both intact protein and free amino acids[6]. The experiment of EWA D. Marczak[7] showed that the hydrolysates of spinach LPC hydrolyzed by pepsin and pancreatin showed anti-blood pressure effect on high-blood pressure mice, but spinach LPC itself had no impact on it. Therefore, in order to improve nutritional and functional properties of protein, protein hydrolysates have been widely used in specific formulation.

2 Materials and Methods

2.1 Materials

Ryegrass LPC: They were self-refined from Ryegrass which was harvested from Fan Chang cow farm (59.93% crude protein, calculated as N%×6.25).

Proteolytic enzyme: The enzyme was alcalase (bought from WuXi), a protease of *Bacillus licheniformis* with endopeptidase activity. The main component of the commercial preparation was Serine protease subtilisin A.

2.2 Calculation of the degree of hydrolysis (DH)

The hydrolysis was carried out using the pH-stat method. DH was calculated as follow:

$$DH\% = (h/h_{tot}) \times 100\% = (V_1 \times C \times 100\%)/(V_2 \times N \times h_{tot})$$

Where V_1 represented the volume of NaOH required to maintain constant pH in ml; C represented the molarity of NaOH (mol/L); V_2 represented the volume of LPC in ml; N represented the concentration of LPC(g/ml); h_{tot} represented the total number of peptide bonds in the protein substrate (mmol/g). The h_{tot} was calculated by using formaldehyde titration method described by Zhao Xinhuai[8].

2.3 Molecular weight determination

The average molecular weight was determined with HPLC. The molecular weights were determined by comparing the retention times with those of standard peptides in a TSKgel 2000 SWXL300 nm×7.8 mm column in Waters 600 HPLC(allocating 2487 UV detector and M32 work station). The following standard peptides or proteins were used: cytochrome C (MW=12500), bacitracin (MW=1450), Gly-Gly-Tyr-Arg (MW=451) and Gly-Gly-Gly (MW=189). A buffer in ratioacetonitrile:water:trifluoroaceticacid=45:55:0.1(V:V:V) was used as the elution solvent with a flow rate 0.5 ml/min. The temperatuer of the column was 30℃. The peptides in the effluent were monitored with a UV detector set at 220 nm.

2.4 Amino acid analysis

Amino acid analysis was performed using ion exchange chromatography following the release of amino acid from protein by hydrolyzing 60 mg of the sample mixed with 8 ml of 6NHCl. The hydrolysation was done under vacuum at 110 ℃ for 24 h. After cooling the hydrolysate was washed in distilled water filter and dried in a rotary evaporator at 60 ℃. The dried samples were then dissolved in 0.01 N HCl. The amino acids in hydrolysate were separated and quantified by injecting 5μl into a Hitachi 835-50 amino acid analyzer equipped with a 2.6×150 mm ion exchange column. The column temperature was 53 ℃. Sodiumcitrate buffer was used as eluent with a flow rate of 0.225 ml/min. The light absorbance of amino acid was detected at 570 nm and the amino acids were quantified by comparing them with amino acid profiles from external amino acid standard.

3 Results and Discussion

3.1 Effect of E/S on DH

The effects of E/S on the DH of ryegrass LPC were shown in Fig. 1.

In general, DH increased with increase in E/S. When E/S was 1750 U/g, DH increased markedly. However, further increase in E/S, the DH increased slowly. So E/S was

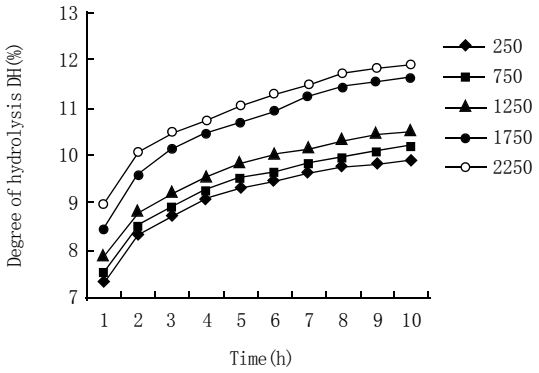

Fig.1 Effects of E/S on DH

determined as 1750 U/g initially.

3.2 Effects of temperature on DH

The effects of temperature on the DH of ryegrass LPC were shown in Fig. 2.

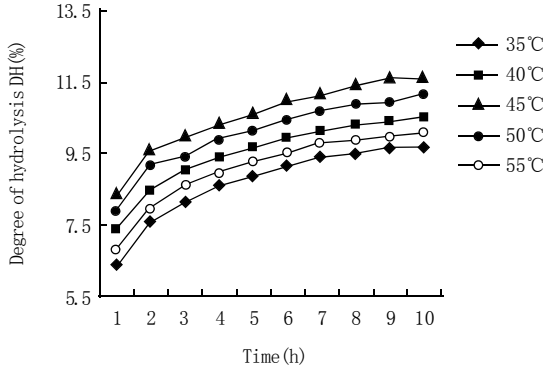

Fig.2 Effects of temperatures on DH

As shown in Fig. 2, when temperature was 45 ℃, DH increased markedly. If further increase in the temperature, DH declined on the contrary. So the optimal temperature for the hydrolysis of ryegrass LPC was 45 ℃.

3.3 Effects of pH on DH

The effects of pH on the DH of Ryegrass LPC is shown inFig. 3.

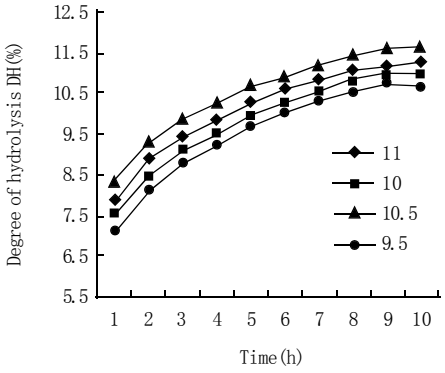

Fig.3 Effects of pH on DH

As shown in Fig. 3, when pH value was 10.5, DH increased markedly. For further increase in pH, DH decreased on the contrary. So the optimal pH for the hydrolysis of ryegrass LPC was 10.5.

3.4 Orthogonal test

According to the above results achieved from a single factor assay, the hydrolysis conditions including E/S, pH, temperature and reaction time were further optimized with an orthogonal layout $L_9(3^4)$. The effects of the four factors on DH were shown in Table 2.

Table 1 Design factors and levels in experiments

Level	Factor			
	E/S	pH	Temperature(℃)	Reaction time(h)
1	1250	10.0	40	8
2	1750	10.5	45	9
3	2250	11.0	50	10

Table 2 $L_9(3^4)$ orthogonal test results

Serial number	E/S	pH	Temperature(℃)	Time(h)	DH (%)
1	1	1	1	1	8.00
2	1	2	2	2	11.20
3	1	3	3	3	10.30
4	2	1	2	3	11.46
5	2	2	3	1	11.50
6	2	3	1	2	9.65
7	3	1	3	2	8.84
8	3	2	1	3	10.07
9	3	3	2	1	11.35
K_1	29.50	28.30	27.72	30.85	
K_2	32.61	32.77	34.01	29.69	
K_3	30.26	31.30	30.64	31.83	
k_1	9.83	9.43	9.24	10.28	
k_2	10.87	10.92	11.34	9.90	
k_3	10.09	10.43	10.21	10.61	
R	1.04	1.49	2.10	0.71	

Note: Relationship among major factors $R_T > R_{pH} > R_{E/S} > R_t$.

Table 3 Variance-time analytical tables with assay of pH-stat method

Variance source	Sum of square	Degree of freedom	Mean square	F Value	Notability
A	1.76	2	0.88	2.28	
B	3.46	2	1.73	4.49	△
C	6.61	2	3.30	8.58	△
D	0.77	2			
Error	0.00	0	0.38		
Correct total	12.59	8			

Note: $F_{0.01}(2,2)=99.00$, $F_{0.05}(2,2)=19.00$, $F_{0.10}(2,2)=9.00$, $F_{0.25}(2,2)=3.00$.

If: $F > F_{0.05}$ Factors were significant specially **

$F_{0.05} \geq F > F_{0.10}$ Factors were notable *

$F_{0.10} \geq F > F_{0.25}$ Factors had an impact on index △

$F_{0.25} \geq F$ Factors had no effect on index

It was obvious from the analysis of capacity and

variance-time above orthogonal test. The relationship with major factors was $R_T > R_{pH} > R_{E/S} > R_t$. The optimum combination of the hydrolysis condition was T_2pH_2E/S_2t_3. The optimal hydrolysis conditions were 45 ℃, pH10.5, and E/S 1750 U/g and 10 h for alcalase hydrolysis of ryegrass LPC. The most important factor was the temperature.

3.5 Molecular weight distribution

A collection of illustrative plates with High Performance Liquid Chromatography was shown in Fig.4.

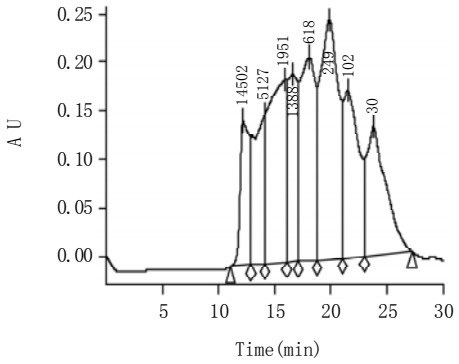

Fig.4 Collection of illustrative plates of RLPC by HPLC

Table 4 Different molecule weight peptides included in RLPC hydrolysates

Retention time of peak(min)	M P	Area(%)
11.067～12.900	14502	6.64
12.900～14.133	5127	7.82
14.133～16.067	1951	15.48
16.067～17.117	1388	9.12
17.117～18.783	618	14.95
18.783～21.033	249	21.13
21.033～22.983	102	12.58

The distribution of relative molecular weights of hydrolysates could be shown in Fig.4. The proportion of peptides with different molecular weight is shown in Table 4. There were 7 kinds of peptides in the protein hydrolysates by HPLC. Their relative molecular weights were between 102 and 14502 while most of them were 249 accounted for 21.13%.

3.6 Amino acids composition

The contents of amino acid in hydrolysates were shown in Table 5.

The profiles of Automatic Amino Acid Analyzer (AAAA) showed that the total content of free amino acid in hydrolysates was 3.233% and the proportion of essential amino acids to total free amino acids was 30.776%. The proportion of sapor amino acids to total free amino acids was 38.324%, and the proportion of drug-effective amino acids to total free amino acids was 75.286%.

Table 5 Contents of amino acid in hydrolysates

Amino acid	Content in hydrolysates(%)	Amino acid	Content in hydrolysates(%)
Asp#	0.455	Phe*#	0.222
Glu#	0.784	Ile*#	0.111
Ser	0.191	Leu*#	0.412
His#	0.003	Lys*#	0.078
Hly#	0.122	Pro	0.221
Thr*#	0.093	T	3.233
Arg#	0.075	E	0.995
Ala	0.283	F	1.239
Tyr	0.088	V	2.434
Cys-s	0.016	E/T	30.776
Val*#	0.005	F/T	38.324
Met*#	0.074	V/T	75.286

Note: *represented necessary amino acids. # represented drug-effective amino acids. *T* represented total content of amino acids. *E* represented content of necessary amino acids. *F* represented sapor amino acids, included asp and glu. *V* represented content of drug-effective amino acids.

4 Conclusion

The optimal conditions for hydrolysis of ryegrass LPC with alcalase were obtained. Temperature at 45 ℃, pH at 10.5 and E/S at 1750 units per gram were beneficial to hydrolysis of ryegrass LPC with alcalase. There were 7 kinds of peptides in the protein hydrolysates. Their relative molecular weights were between 102 and 14502. Most of them were 249 which accounted for 21.13%. The total content of free amino acids in hydrolysates was 3.233%. The proportion of essential amino acids, sapor amino acids and drug-effective amino acids to total free amino acids were 30.776%, 38.324% and 75.286%, respectively.

It is obvious that the enzymatic hydrolysis is a way to improve the functional and nutritional properties of the products and it is of great importance with respect to food application of protein hydrolysates.

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以乳清为原料酶法水解乳糖条件的研究

徐 宁，孙 波，迟玉杰*
（东北农业大学食品学院，黑龙江 哈尔滨 150030）

摘 要：乳糖溶解性差、甜度低，加之部分人群具有乳糖不耐症等原因，乳清中乳糖的利用率相对较低。利用β-半乳糖苷酶对乳清中的乳糖进行水解，既可以改善乳糖的溶解性能和甜度，又有利于提高乳清中乳糖的利用率。本实验通过研究乳清中乳糖的浓度、β-半乳糖苷酶的使用量、水解液温度、水解液pH对乳糖水解度的影响，确定了以乳清为原料酶法水解乳糖的适宜条件，即乳糖浓度15%、β-半乳糖苷酶的使用量为90U/g乳糖、水解液温度40℃、水解时间3h、水解液pH值6.5，在此条件下水解度为68%。

关键词：乳清；乳糖；水解；乳糖酶

Study on Enzymatic Hydrolysis of Lactose in Whey

XU Ning, SUN Bo, CHI Yu-jie*
(Food College, Northeast Agricultural University, Harbin 150030, China)

Abstract: Bad solubility, low saccharinity of lactose and lactose intolerance have caused low availability of whey. Now β-galactosidase was applied for hydrolysis of lactose in whey. The treated lactose in whey improved saccharinity and solubility thus easily utilized. Papers studied the effects of initial lactose concentrations and enzyme dosage, temperature and pH on degree of lactose hydrolysis. Orthogonal test was designed to identify optimum hydrolysis conditions for yielding most monomeric saccharides. The optimum conditions of hydrolysis for lactase was that substrate concentration 15%, 40 ℃, 3 h and of lactase dosage 90 U/g. Under these conditions the hydrolysis degree is 68%.

Key words: whey; lactose; hydrolyze; lactase
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乳清是生产干酪、干酪素的副产品，它含有鲜乳中55%的营养成分，每生产1kg干酪要排放9kg乳清。2005年世界乳清产量大于14670万吨，有效利用率不到50%。尽管目前我国的乳清产量较少，但随着干酪产量的不断提高，乳清产量将不断增加。大量的乳清排放既造成资源浪费，又导致严重的环境污染[1]。乳清的处理和综合利用依然是乳品工业的一个重要问题，因此，迫切需要寻找一种有效的方法利用乳清。

乳清含有的碳水化合物有98%以上为乳糖[2]。目前乳清中的乳糖用作食品添加剂，添加到糖果、糕点及各种食品中，但是由于乳糖的溶解性差，甜度低以及部分人群对乳糖具有不耐性等原因，乳糖的添加影响了产品的风味和加工性能[3]。

β-半乳糖苷酶应用于水解牛奶和乳清中的乳糖[4]，乳糖在β-半乳糖苷酶的作用下分解成葡萄糖和半乳糖，水解后得到的混合糖浆的甜度较乳糖提高4～5倍，溶解

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作者简介：徐宁(1981-)，女，硕士研究生，研究方向为发酵工程。

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