J. KOREAN SOC. FOOD NUTR.
17(2) 136~143(1988)

Conditions of Quantitative Analysis for Free Amino Acid in Fermented Proteins

Hong-Soo Ryu, Jung-Hye Moon and Kang-Ho Lee*

Dept. of Nutrition and Food Science, National Fisheries University of Pusan, Pusan, 608-023, Korea

**Dept. of Food Science and Technology, National Fisheries University of Pusan, Pusan, 608-023, Korea*

발효단백질의 유리아미노산 정량

류홍수 · 문정혜 · 이강호*

부산수산대학 식품영양학과. 부산수산대학 식품공학과*

Abstract

This study was performed to provide the optimal conditions of quantitative analysis for free amino acid in fermented protein foods. The water extractable free amino acid from dairy fermented foods was extracted effectively at 75℃ for 40 min., while it were extracted from fermented soy products at 40℃ for 3 hours. A close results of free amino acid content to those from amino acid analyzer were obtained using OPDA method with lysine standard after deproteinizing with 1% picric acid. 95% ethanol used as a deproteinizing reagent could give a comparable results to those from picric acid treatment in determining free amino acid content using OPDA method. Therefore, ethanol treatment was more recommendable than picric acid treatment which has some troubles in removing excess picric acid through Dowex resin column. The most desirable precipitation method for free amino acid determination using TNBS method was 95% ethanol treatment among the various deproteinizing procedure. The copper salt method was not suitable owing to its lacking reproducibility and pronounced discrepancy in determining free amino acid.

INTRODUCTION

Fermented foods are important components of the diets of people throughout the world. In many cases the final products make major contributions to the diets as source of protein, calories, mineral and some vitamins[1], and those protein products may be grouped into three categories such as fermented dairy, soybean and fish products. In general, those are more attractive to the consumers than the raw ingredients due to the organoleptic characteristics. Fermentation does not increase the protein nutritive value but the more amino acids are released and digestibility is improved over the raw ones[2].

Therefore the protein nutritive value of fermented protein foods is dependent on the free amino acid content in it. But it is true that the determined content of free amino acid is varied with methods adopted. This research was undertaken to establish a condition of quantitative method that can give a close result in comparison with those from automatic amino acid analyzer.

MATERIALS AND METHODS

Sample Preparation

Cheeses used in this study(processed cheese, substitute cheese, aged cheese A and B) were given by Fisher Cheese Co.(Wapakoneta, OH, USA). The other fermented dairy products(plain yoghurt and butter milk) and fermented soy products(Koeran soy paste, Japaness soy paste and black bean paste) were purchased from local food store. Cheese products and soy products were dried in a hot air blast dryer at 80℃ for 6 hours. Decantation was taken to lower the fat content that can cause trouble in further grinding and sieving followed after drying. Additional fat extraction from cheese products at room temperature was carried out using 95% ethanol for 12 hours and 95% n-hexane for 24 hours. The remained organic solvents were blew off in a draft and the defatted sample was ground in Waring Commercial Mill to pass a 50 mesh screen. The plain yoghurt and butter milk were frozen in -40℃ deep freezer for 2 days and then thawed at room temperature. After thawing, the supernatant fraction of those products were discarded for easy freeze drying in Virtis type freeze dryer. The ground samples were placed in plastic bags and stored at -15℃ until needed.

Extraction and Deproteinizing

Water extractable free amino acids from fermented protein foods were prepared as previously described[3,4] with slight modifications. An approximately 0.2 g (soy products) and 0.5 g (dairy products) of samples were dispersed with agitation in 50ml distilled water at 40℃ for 2 hours(soy products) and at 75℃ for 40 minutes(dairy products) in a shaking water bath. Protein dispersions were cooled to room temperature and were filtered through whatman No. 41 filter paper(11cm). To 15ml filtrate 45ml 95% ethanol were added and then mixture kept overnight at 7℃ for precipitation to take place. Deproteinization with 1% picric acid was carried out by a procedure similar to that of Stein and Moor[5]. Heating procedure, on 100℃ water bath for 3 min., was employed to precipitate proteins in the extracted solution also.

Analysis of Free Amino Acids

Free amino acid was determined using o-phthaldialdehyde spectrophotometric assay(OPDA method) given previously[6,7]. OPDA reagent was prepared just prior to use by mixing 25ml 0.05M borate buffer(pH 9.7) with 0.4ml 1% OPDA soln. (w / v) in ethanol and 0.4ml 1% mercaptoethanol (v / v) in ethanol. To determine the content of free amino acid, 0.8ml filtrate was added to directly to 0.8ml of OPDA reagent in a 3ml quartz cuvette and the solution was mixed briefly by inversion at ambient temperature. Read the absorbance of solution at 340nm against a water blank after 10 minute color development using Beckman DU-6 Spectrophotometer. TNBS(2,4,6-trinitrobenzenesulfonic acid) method showed in Kakade and Liener,[8] Hall et al.,[9] and James and Ryley[10] was used as the starting point of this assay with some modification. 1ml of deproteinized solution was treated with 1ml of 0.01 M phosphate buffer(pH 8.15) followed by 1ml of 0.01% TNBS solution, and incubted in a water bath at 60℃ for 1 hour. After incubation, 1ml of 0.1 N HCl was added into color developed solution and agitated it vigorously. Prior to check O.D. at 420nm, the solution was allowed to stand for 30 minutes at room temperature. Copper salt method of Spies Chamber[11] was used in determining the content of free amino acid also. The standard curves with D.L.-lysine and D.L.-leucine for OPDA method and TNBS method were made as the methods shown in earlier paper.[7,10] The amino acid profile of deproteinized samples, treated with 1% picric acid, was determined

in a Beckman 120C type automatic amino acid analyzer.

RESULTS AND DISCUSSION

Determining the content of water extractable free amino acid from fermented protein foods is influenced severely by the extraction temperature and length. Water extraction of free amino acid was routinely performed at temperature range of 40℃-80℃ for from 10 minutes to 5 hours as stated in previous reports.[12~15] According to the results from preliminary experiments for optimal condition, two kinds of extracting procedure(40℃, 3 hours and 75℃, 40 minutes) were done for water extraction of free amino acid from fermented protein foods.

The results in Table 1 showed that extracting at 40℃ for 3 hours was more effective than that at 75℃ for 40 min. in case of fermented soy protein but the more free amino acid was determined at later condition than the former condition in fermented dairy products. It might be indicated that mild heating caused an unfolding of protein and it allowed a more free amino acid from fermented soy proteins. Profiles of water extractable free amino acids from fermented foods are shown in Table 2. It can be seen that very slight difference exist in amino acid profiles and total levels between dried ones in this study and fresh cheeses in previous reports.[14,15] On other hand, it was noted the lower level in the total quantities of indivdual amino acids in yoghurt and butter milk than in other reports.[16~18] The striking difference betwen the data of free amino acid contents in yoghurt and butter milk may be related to a great loss of free amino acid during sample preparation as described in previous chapter of this paper. The total free amino acid content of fermented Korean soy paste was not affected by a heat treatment(Table 2) but the hot air blast dried soy products(Japaness soy paste and black bean paste) showed extremely low levels free amino acids as compared to that of freeze dried ones(Table 1 and 2). It might be expected that the nonenzymatic browning could have been resulted the lower free amino acid levels during hot air drying.

The total free amino acid determined using OPDA method with lysine standard could offer a close results in comparison with the results from amino acid analyzer as shown in Table 3. About two times of total free amino acid content using OPDA

Table 1. variations in free amino acid contents[a] using two kinds of water extracting procedure (% dry basis)

Sample[b]	40℃, 3 hrs		75℃, 40 min	
	LEU[d]	LYS[d]	LEU	LYS
1. ANRC Casein	0.124	0.052	0.197	0.079
2. Processed Cheese	1.099	0.483	3.833	1.767
3. Substituted Cheese	2.439	1.107	4.256	1.816
4. Aged Cheese A	4.438	2.042	2.191	1.938
5. Aged Cheese B	4.580	2.106	4.483	2.702
6. Plain Yoghurt[c]	1.337	0.608	1.908	0.682
7. Butter Milk[c]	0.664	0.298	0.771	0.346
8. Korean Soy Paste	28.770	13.307	7.448	3.451
9. Japanese Soy Paste	8.928	4.096	7.249	3.150
10. Black Bean Paste	8.474	3.888	7.037	3.262

[a] Determined using OPDA method after ethanol deproteinizing.

[b] Hot air blast dried products except for No. 1, 6 and 7.

[c] Discarded supernatant fractions in thawed products before freeze drying

[d] LEU : Determined as equivalent of DL-leucine LYS : Determined as equivalent of DL-lysine

Table 2. Concentration(g/100g solid) of individual free amino acids in fermented protein foods[1,2)]

Amino acid	No.2	No.3	No.4	No.5	No.6	No.7	No.8	No.10	No.11	No.12	No.13	Whole Egg
LYS	0.150	0.058	0.247	0.235	0.026	0.009	0.766	0.064	1.421	0.470	0.147	0.039
HIS	0.003	trace	0.004	0.034	trace	0.002	0.023	trace	0.150	0.050	0.014	0.008
NH_3	0.169	1.086	0.122	0.150	0.405	0.055	0.603	0.045	0.121	0.238	0.221	0.030
ARG	0.018	trace	0.029	0.005	0.014	0.005	0.151	0.063	0.061	0.534	0.192	0.034
ASP	0.043	0.037	0.044	0.069	0.031	0.008	0.313	0.099	0.570	0.041	0.112	0.025
THR	0.044	0.024	0.050	0.056	0.007	trace	0.348	0.152	0.455	0.269	0.125	0.027
SER	0.098	0.053	0.101	0.108	0.023	0.003	0.557	0.045	0.514	0.284	0.122	0.036
GLU	0.193	0.913	0.110	0.284	0.007	0.047	1.543	0.027	1.419	0.450	0.155	0.043
PRO	trace	trace	0.083	0.132	0.055	0.023	0.627	0.037	0.614	0.275	0.209	0.018
GLY	0.024	0.039	0.026	0.031	0.006	0.001	0.290	0.012	0.409	0.152	0.068	0.010
ALA	0.062	0.051	0.059	0.061	0.015	0.003	1.137	0.051	1.056	0.289	0.132	0.013
VAL	0.117	0.023	0.165	0.139	0.016	trace	0.661	0.105	0.724	0.326	0.136	0.023
MET	0.031	trace	0.020	0.037	trace	trace	0.058	trace	0.278	0.176	0.033	trace
ILE	0.039	0.007	0.045	0.053	0.007	trace	0.685	0.101	0.717	0.350	0.140	0.020
LEU	0.228	0.108	0.267	0.260	0.023	0.003	1.102	0.095	1.267	0.635	0.233	0.039
TYR	0.063	0.038	0.029	0.071	0.008	0.005	0.348	0.117	0.393	0.301	0.104	0.007
PHE	0.157	0.065	0.217	0.210	0.009	0.004	0.545	0.060	0.761	0.378	0.139	0.027
Total	1.439	2.502	1.628	1.935	0.652	0.168	9.757	1.073	10.930	5.578	2.273	0.399

1) Samples from No.2 to No.10 are indentical as shown in Table 1.

2) No.11, No.12 and No.13 are freeze dried products for Korean soy paste, Japanese soy paste and black bean paste.

Table 3. Content of water extractable free amino acid determined by OPDA method[a] (% dry basis)

Sample	Amino acid analyzer[b]	LEU[c]	LYS[c]
1. ANRC Casein	None	0.147	0.057
2. Processed Cheese	1.440	3.606	1.647
3. Substituted Cheese	2.502	5.679	2.614
4. Aged Cheese A	1.623	4.460	2.045
5. Aged Cheese B	1.936	4.966	2.284
6. Plain Yoghurt	0.652	1.511	0.689
7. Butter Milk	0.169	0.825	0.372
8. Korean Soy Paste	9.756	24.084	11.146
9. Japaness Soy Paste	N.D.	8.795	4.045
10. Black Bean Paste	1.073	8.319	3.826
11. Freeze Dried Korean Soy Paste	10.929	25.355	11.731
12. Freeze Dried Japanese Soy Paste	5.576	17.309	8.196
13. Freeze Dried Black Bean Paste	2.276	10.423	7.761
14. Freeze Dried Whole Egg	0.400	4.710	2.173

[a] Using ethanol(95%) precipitating procedure

[b] Deproteinizing with 1% picric acid and then passed through Dowex resin column.

[c] LEU and LYS : Determined as equivalent of DL−leucine and DL−lysine, N.D. : Not determined
Sample No.1~No.7 : Extraction was done at 75℃ for 40 min.
Sample No.8~No.14 : Extraction was done at 40℃ for 3 hrs.

Table 4. Influence od deproteinizing procedure on the free amino acid content determined using OPDA method

(% dry basis)

Sample[a]	Amino acid Analyzer[b]	Ethanol (95%)	Heating (100℃, 3 min)	Picric acid (1%, w/v)
		LYS[c]	LYS[c]	LYS[c]
No. 1.	None	0.061	0.064	0.075
2.	1.140	1.740	1.897	1.626
3.	2.502	2.352	2.228	2.610
4.	1.628	2.049	2.257	2.117
5.	1.936	2.204	2.541	2.113
6.	0.652	0.654	0.970	0.800
7.	0.169	0.347	0.418	0.407
8.	9.756	12.000	13.237	12.216
9.	N.D.	3.896	3.951	2.727
10.	1.073	3.827	3.951	1.625
11.	10.929	11.731	12.056	10.934
12.	5.576	8.196	7.981	8.202
13.	2.272	4.761	4.364	3.100
14.	0.400	2.173	2.315	0.367

[a] Samples are same as detailed in Table 1 and Table 3.

[b] Deproteinized with 1% picric acid and then passed through Dowex resin column.

[c] LYS : Determined as equivalent of DL-lysine N.D. : Not determined

Table 5. Free amino acid content determined using TNBS method[a]followed after various deproteinizing procedure

(% dry basis)

Sample[b]	Amino acid analyzer[c]	Ethanol (95%)	Heating (100℃, 3min)	Picric Acid (1%, w/v)
No. 1.	None	0.214	0.598	N.D.
2.	1.440	7.898	8.317	N.D.
3.	2.502	9.262	9.447	N.D.
4.	1.628	6.040	6.556	N.D.
5.	1.936	6.843	7.368	N.D.
6.	0.652	2.000	2.148	N.D.
7.	0.169	0.942	1.264	N.D.
8.	9.756	27.912	37.077	0.284
9.	—	9.495	11.830	0.303
10.	1.073	5.982	11.728	0.309
11.	10.929	24.468	26.213	N.D.
12.	5.576	16.387	18.540	N.D.
13.	2.272	10.505	14.465	N.D.
14.	0.400	5.994	5.511	0.004

[a] Determined as equivalent of DL-leucine[b] Samples are same as detailed in Table 1 and 3.

[c] Deproteinized with 1% picric acid and then passed through Dowex resin column. N.D. : Not detected

method with leucine standard could be explained by the number of with OPDA reagent. amino groups in lysine and leucine. Comparisons of free amino acid contents in fermented protein foods, determined using OPDA method with various deproteinizing procedure such as 95% ethanol, 1% picric acid treatment and heating(100℃, 3 min), indicated that the results gained through picric acid treatment were closer to results from amino acid analyzer than the other treatment consistently (Figure 1 and Table 4). Moderate results could be obtained with 95% ethanol treatment also, and this treatment was more recommendable than 1% picric acid treatment which has an additional step to remove the excess picric acid, employing Dowex resin column.

Content of free amino acid determined using TNBS method with leucine standard was varied with the adopted deproteinizing procedure(Tabe

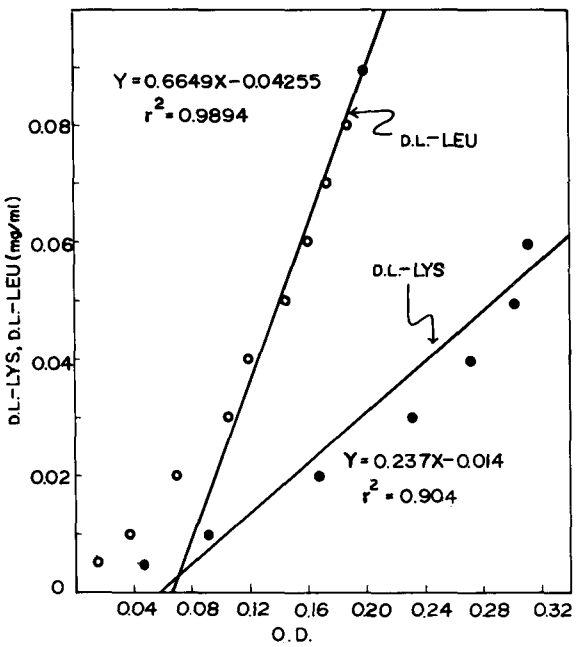

Fig. 1. Relationship between the concentration of D.L.-lysine aud D.L.-leucine, and optical density after reaction of OPDA(o-phthaldialdehyde).

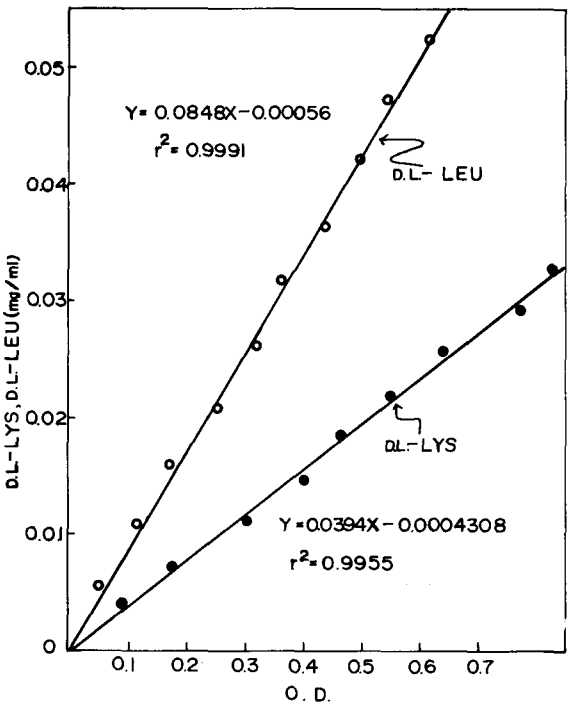

Fig. 2. Standard curve on absorbance vs D.L.-lysine and D.L.-leucine after OPDA (o-phthal dialdehydeo) color developing.

5 and Figure 2). Among the deproteinizing procedures showed in Table 5, 95% ethanol treatment was most effective but this treatment resulted high levels of free amino acid in comparison with OPDA method(Table 3). When using 1% picric acid treatment as a deproteinizing step in TNBS method, free amino acids were not detected in almost of samples due to the interfering effect of picric acid on color developing wth TNBS reagents[10]. In Table 6, the quantitative results of free amino acid content using copper salt method[11] was compared to the results from the other color developing method. The data in Table 6 show that copper salt method lacked in consistency, and reproducibility in determining free amino acid content for fermented protein foods. Therefore, it might be expected that copper salt mthod is not suitable in performing free amino acid determination.

Table 6. Variations in free amino acid content after different color developing procedure (% dry basis)

Sample[a]	Amino Acid Analyzer[b]	ethanol precipitation (95%)			Heat precipitation (100℃,3min)		
		OPDA	TNBS	Copper-salt	OPDA	TNBS	Copper-salt[c]
		LYS	LYS	ALA	LYS	LYS	ALA
No. 1	None	0.06	0.08	0.27	0.06	0.22	0.05
2	1.44	1.74	3.63	4.79	1.90	3.83	1.22
3	2.50	2.35	3.34	4.87	2.23	4.35	2.72
4	1.62	2.05	2.78	1.55	2.26	3.02	0.99
5	1.94	2.20	3.15	1.66	2.54	3.39	0.58
6	0.65	0.65	0.92	1.03	0.97	0.99	0.33
7	0.17	0.35	0.43	0.50	0.42	0.58	0.12
8	9.76	12.00	12.84	8.53	13.24	17.06	2.43
9	—	3.90	4.37	10.45	4.00	5.44	1.71
10	1.07	3.83	2.75	10.62	3.95	5.40	1.64
11	10.93	11.73	11.26	8.07	12.06	12.06	2.54
12	5.58	8.20	7.54	14.67	7.98	8.53	2.50
13	2.27	4.76	4.83	9.86	4.36	6.65	1.28
14	0.40	4.71	2.76	3.96	2.32	3.92	0.16

[a] Sample are same as detailed in Table 1 and Table 3.

[b] Deporoteinized with 1% picric acid and then passed through Dowex resin column.

[c] Copper-salt : Copper-salt method of Spies and Chamber(1958)

요 약

발효단백질 중의 유리아미노산을 효과적으로 정량하기 위하여 정량방법(추출조건, 除蛋白 및 발색법)에 따른 영향을 검토하였다. 낙농발효품의 수용성 유리아미노산은 75℃, 40분, 대두발효품은 40℃, 3시간에서 효과적으로 추출되었다. 여러 침전방법(95% ethanol 처리, 100℃, 3분 가열 및 1% picric acid 처리) 중 1% picric acid로 제단백하고 OPDA(*o*-phthaldialdehyde)로 발색시켜 D.L.-lysine을 표준아미노산으로 삼아 정량, 계산하였을 때가 아미노산자동분석기에 의한 결과에 가장 접근하였으나 ethanol로 침전시킨 뒤 OPDA로 발색시킨 결과도 이와 유사하였다. TNBS(2,4,6-trinitrobenzensulfonic acid)에 의한 발색정량에는 ethanol 침전처리가 가장 효과적이었으나 종래의 銅鹽法(copper salt method, Spies and Chamber, 1958.)에 의한 결과는 다른 비색정량 및 아미노산자동분석기에 의한 결과에 비하여 시료에 따른 과소의 차이가 심하였고 재현성이 결여되었다.

ACKNOWLEDGEMENT

This study has been supported by the Korean Science and Engineering Foundation as Post-Doctoral Research Program from 1986 to 1987.

REFERENCES

1. Pederson, A.S : Nutritious Fermented foods of the Orient, in "*Micrbiology of Food Fermentation*", AVI Pub. Co., Westport, Connecticut, pp.231-246(1971)
2. Beuchat, L.R. : Traditional Fermented Food Products, in "*Food and Beverage Mycology*", AVI Pub. Co., Westport, Connecticut, pp. 224-256(1976)
3. Kosikowsky, F.V. : *J. Dairy Sci.*, **34**, 228(1951)
4. AOAC : "*Official Methods of Analysis*", 13th ed., Association of Official Analytical Cemists, Washington D.C., pp.224(1976)
5. Stein, W.H. and Moor, S. : *J. Biol. Chem.*, 211, 915(1954)
6. Rowlet, R. and Murphy, J. : *Analytical Biochem.*, 112, 163(1981)

7. Church, F.C., Swaisgood, H.E., Porter, D.H. and Catignani, G.L. : *J. Dairy Sci.*, **66**, 1219 (1983)
8. Kakade, M.L. and Liener, I.V. : *Analytical Biochem.*, **27**, 173(1969)
9. Hall, R.J., Trinder, N. and Givens, D.I. : *Analyst*, **98**, 673(1973)
10. James, N.A. and Ryley, J. : *J. Sci. Food Agric.*, **37**, 151(1986)
11. Spies, J.R. and Chamber, D.C. : *J. Biol. Chem.*, **191**, 787(1958)
12. Noomen, A. : *Neth. Milk Dairy J.*, **31**, 210(1977)
13. Yong, F.M. and Wood, B.J.B. : *J. Food Tech.*, **12**, 163(1977)
14. Visser, F.M. : *Neth. Milk and Dairy J.*, **31**, 210 (1977)
15. Kuchroo, C.N. and P.F. Fox : *Milchwissenschaft*, **37**, 542(1982)
16. Rasic, J., R.S., C.T. and Obradovic, B. : *Milschwissenschaft*, **26**, 497(1971)
17. Breslaw, E.S. and Kleyn, D.H. : *J. Food Sci.*, **38**, 1016(1973)
18. Deeth, H.C. and Tamime, A.Y. : *J. Food Protec.*, **44**, 78(1981)
19. Satake, K., T. Okuyama, M.O. and Shinoda T. : *J. Biochem.*, **47**, 654(1960)

(Received May 20, 1988)