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Masatake Hori, Isamu Aoki, and Satoru Kuwada : Amino Acids in the Mycelium of *Eremothecium ashbyii*. (Application of Chromatography. XLVII.*1).

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Kuwada, *et al.*[1] previously investigated the free amino acids in the $V.B_2$-producing yellow-type strain and the not $V.B_2$-producing leuco-type strain of *Er. ashbyii* by two-dimensional paper chromatography, and obtained the results that hydroxykynurenine was (+) and proline was (−) with the former strain and hydroxykynurenine was (−) and proline was (+) with the latter strain. They reported that the difference might be due to the significant difference in amino acid metabolism of the two strains. But the comparison of the two strains by this data was insufficient because samples were small in quantity and it was merely a qualitative test. In the present study the authors reexamined the previous investigation by using an automatic amino acid analyzer.

Table I. Analytical Results through Amino Acid Analyzer (mg./g. wet mycelium)

Amino acids	Yellow-type strain			Leuco-type strain		Remarks
	Sample (a)	Sample (b)	(b)/(a)	Sample (c)	(a)/(c)	
Lysine+Ornithine	1.04	0.52	0.50	8.01	0.13	
Histidine	1.55	0.23	0.15	1.27	1.22	
Ammonia	0.15	0.25	1.67	0.21	0.71	undetected by PPC
Arginine	3.52	1.99	0.57	0.88	2.50	〃
Tryptophan	0.39	0.16	0.41	0.12	3.08	
Aspartic acid	0.51	0.11	0.55	ca. 0.3[a]	1.7[b]	
Threonine	0.61	0.16	0.38	0.38	1.61	
Serine	1.63	0.60	0.37	0.61	3.74	
Glutamic acid	1.94	2.44	1.25	ca. 0.6[a]	3.2[b]	
Proline	0.64	0.75	1.17	0.33	1.94	
Glycine	0.32	0.26	0.81	0.79	0.41	
Alanine	1.53	1.26	0.82	0.40	2.52	
Cystine	0	0		0.41	—	undetected by PPC
Valine	0.80	0.20	0.25	0.28	2.86	
Methionine	0.29	0.09	0.31	0.13	2.23	undetected by PPC
Isoleucine	0.67	0.10	0.15	0.21	3.19	〃
Leucine	1.02	0.18	0.18	0.36	2.83	
Tyrosine	1.17	0.15	0.13	1.24	1.06	
Phenylalanine	0.64	0.10	0.16	0.27	2.37	
Hydroxykynurenine	0.61	0.28	0.46	0.26	2.34	

a) Overlapped to unknown peak. *b*) Insufficient data because of its overlapping.

The two strains preserved in the Technical Division of Takeda Chemical Industries, Ltd. and the Institute for Fermentation respectively were cultivated in a polypeptone medium under the same conditions as before.[1] The mycelium was extracted with water at 80° and filtered again. The extracts, after being concentrated *in vacuo*, were applied to two-dimensional paper partition chromatography. The behaviors of the extracts to the ninhydrin reaction were about the same as before, but in the present experiment, proline

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1) M. Asai, T. Masuda, S. Kuwada : This Bulletin, **9**, 496 (1961).

was detected in the yellow-type strain. And, when the chromatograms were observed under ultraviolet rays before being treated with ninhydrin, hydroxykynurenine was detected as a fluorescent spot in the yellow-type strain but not at all in the leuco-type strain. From the results it is concluded that i) the amino acid metabolism of the strains seems different according to culture conditions, and ii) as there is a limit in sensitivity in the detection by two-dimensional paper partition chromatography, it seems significant to conduct the quantitative determination by a more sensitive method.

In the present study, Hitachi automatic amino acid analyzer KLA-2 was used. The results of investigation of the free amino acids in the yellow- and leuco-type strains are given in Table I. Of the two strains, the yellow-type strain was extracted with water at 80° and the extract was concentrated to give sample (a), and on the other hand, the same strain was extracted with a homoblendor under cooling and the extract was freeze-dried to yield sample (b). The leuco-type strain was extracted and processed in the same manner as (a) to give sample (c), and these results were compared from the values of (b)/(a) and (a)/(c).

When the results in the present study were compared with those in the two-dimensional paper partition chromatography, arginine, cystine, methionine, isoleucine, and ammonia, which were not detected in the latter method, were observed in the former. And of these amino acids, arginine was unexpectedly large in quantity. The comparison of (a) with (b) revealed that they were nearly the same in the kinds of amino acids, but (b) was lower than (a) in the quantity of amino acids except ammonia, glutamic acid and proline. This may be due to the fact that when the mycelium is extracted with warm water, not only amino acids but also protein, peptides and enzyme such as protease, are extracted, and the amount and kind of amino acids increase during the extraction and concentration of the extract. The reason that ammonia was detected in a large quantity in (b) may be due to the fact that in (b) the extraction was carried out with cooling and then freeze-dried, while in (a) the concentration of the extract was conducted *in vacuo* under warming, so ammonia was driven off during the process. Amounts of glutamic acid and proline were comparatively larger in (b), but the difference was so small that it seemed to be in the limit of deviation of their contents in the mycelium.

However, the most important is the comparison between (a) and (c). The value of (a)/(c) was mostly above one, but proline, which was previously thought to be contained in the leuco-type strain but not in the yellow-type strain, was detected in the present study in the latter strain about two times as much as that in the former. On the other hand, hydroxykynurenine, which was considered to be contained only in the yellow-type strain when investigated by two-dimensional paper partition chromatography, was detected also in the leuco-type strain about ½ time as much as that in the yellow-type strain when investigated with the automatic amino acid analyzer. This seems to be due to the difference between the two methods in sensitivity. Accordingly, it must be recognized that the yellow- and leuco-type strains are not characterized by the presence or absence of proline and hydroxykynurenine.

Experimental

1) **Cultivation of *Er. ashbyii*** ——The $V.B_2$-producing yellow-type strain preserved in the Technical Division of our Industries and the not $V.B_2$-producing leuco-type strain supplied from Prof. Takata's laboratory of School of Technology, Kyoto University, were cultivated in a polypeptone medium for 7 days. And the resulting seed cultures were further cultivated on a large scale for 114 hr. under shaking. Although pH was not adjusted, it was 5.5~6.0 all the time.

2) **Extraction of the Mycelium** ——The mycelium thus obtained was filtered and washed with dist. H_2O 3~4 times until the washing became colorless. The yield of the wet mycelium was 84.3 g. from the yellow-type strain and 103 g. from the leuco-type strain.

Each of these mycelia was extracted with 100 ml. of dist. H_2O at 80° for 15 min. and filtered. The filtered cake was further extracted twice with 60~70 ml. of dist. H_2O under the same conditions as above, and the combined extract was evaporated *in vacuo* to dryness at a temperature lower than 50°. The yield of the solid extract from the yellow-type strain was 8.97 g. and that from the leuco-type strain was 7.13 g.

3) **Comparison of Free Amino Acids in the Two Extracts by Two-dimensional Paper Partition Chromatography**——Each of the above two extracts was dissolved in H_2O to make a ca. 50% (w/v) solution, and about 0.01 ml. of the solution was applied to the starting point on Toyo filter paper No. 51 (40×40 cm.) and developed two-dimensionally with i) BuOH-AcOH-H_2O (4:1:5) and ii) PhOH-H_2O. Pure amino acids were also developed under the same conditions as control. And free amino acids were identified by the ninhydrin reaction and by observation under UV rays. The results were nearly the same as Report XLII of this series except that proline was observed in the yellow-type strain. In the present study, also, the spot of hydroxykynurenine was detected in the yellow-type strain but not in the leuco-type strain. When investigated by the ninhydrin reaction, kynurenine and methionine overlapped each other and hydroxykynurenine piled on tyrosine. Therefore, kynurenine and hydroxykynurenine were observed as fluorescent spots under UV rays.

4) **Determination of Amino Acids with Automatic Amino Acid Analyzer**——The extract obtained from the mycelium of the yellow-type strain as described in 2) was named sample (a) and that obtained from the leuco-type strain, sample (c). When the mycelium is extracted and concentrated by the method described in 2), protein may be decomposed by the action of the enzyme contained in the mycelium, and as a result the quantity and kind of amino acids will increase. So, 17.5 g. of the fresh mycelium of the yellow-type strain was extracted with 30 ml. of H_2O under cooling with ice H_2O, using Nihon Seiki's homoblendor. Incidentally, the blendor was operated at low speed at first for 3 min. and then at high speed (maximum, 20,000 r.p.m.) for 20 sec. The resulting extract was filtered through filter paper and immediately freeze-dried to give about 600 mg. of a residue (b).

Each of these three samples (a), (b) and (c) was dissolved in citrate buffer of pH 2.2 and the amount of the solution corresponding to 10 mg. of the sample was applied on a column of Amberlite CG-120 for acidic and neutral amino acids and on the column for basic amino acids and developed by the usual method. The peaks on the chromatograms thus obtained were calculated by the HW method. The results are summarized in Table I. As expected, the amount of free amino acids in the sample (b) was smaller than that in the sample (a), and the value of (b)/(a) was mostly below one.

Comparison of the yellow-type strain with the leuco-type strain must of course be made with the samples (a) and (c), which were prepared under the same conditions, and examples in which the value of (a)/(c) was below one were very small in number. In other words, the yellow-type strain seems to contain a larger amount of amino acids than the leuco-type strain in general.

Summary

In the previous investigation on the free amino acid contents in the mycelium of *Er. ashbyii*, hydroxykynurenine was (+) and proline was (−) in the yellow-type strain and hydroxykynurenine was (−) and proline was (+) in the leuco-type strain. This time the investigation was reexamined with a little different result that hydroxykynurenine was (+) and proline was (+) in the yellow-type strain and hydroxykynurenine was (−) and proline was (+) in the leuco-type strain. Consequently the amino acids were examined more minutely using automatic amino acid analyzer, and from the results shown in the table it was concluded that the characterization of the two strains by the specific amino acids contained in them is unreasonable.

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