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BIOCHEMICAL STUDIES ON THE RIBOFLAVIN IN GREENBEANS DURING GERMINATION

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Introduction

Greenbeans contain large quantites of nourishments. They are especially abundant in proteins of high biological value and in fats of good qualities. What is more, they are available for a wide variety of preparations and cooking in various forms. They are eaten for food in form of germinating greenbeans. Greenbean sprouts are regarded as one of the staple food and they are the popular main side dish in our country. Since such beans as greenbeans are easily stored up, greenbeans can be made of fresh vegetables by germinating them whenever neccessary (e.g. in winter confinement or on a long voyage).

Generally, during the germination, the greenbeans produce various vitamins e.g. thiamine biotine, riboflavin, pyridoxin, inositol, pantothenic acid, folic acid and ascorbic acid.[1],[2],[3],[4],[5],[6],[7],[8],[9] Furthermore it hasalre ady been shown that the ester of flavin could be synthesized from free rioflavin in the living body[10],[11],[12] and the enzymes for the phosphorylation of free riboflavin and FMN could be purified.[13],[14] It was also known that B-group vitamin contents increased to a large extent during their germination.[6]

Hence greenbean sprouts may be considered as a potential food source. This is interesting and significant from the viewpoint of bichmistry. In spite of the utilization of greenbeans as a soybean, biochemical changes and the changes of nutritional values in the greenbean germination have not been studied well. In this connection, it was attempted to find out scientific principle to deal with this problem by making clear the formation and the changes of the flavin during the green bean germination.

In the present study an attempt was made to observe the changes in its growth and its flavin contents during its germination.

Furthermore, the fractional determination fo three forms of flavin, namely FAD(flavin adenine dinucleotide), FMN(flavin mononucleotide) and FR(free riboflavin) was carried out under adquate conditions.

Experimental Method sand Materials

i) Reagent used

All the reagent used in this study was fully purified before use. And only a brief outline will be given here.

Impurities of chloroform were treated with sulfuric acid for a day, washed twenty times with water, moisture removed with anhydrous sodium sulfate, then distilled at 60°C.

All the other chmicals used were of reagent grade.

ii) Sample and Cultivation of Germinating Greenbeans

Greenbeans (in market) were carefully selected out by sieve.

Cultivation: Park and Kim's method[15] was adopted to prevent molds and degeneration as well as possible to germinate the greenbean averagely all around. The sterilized greebeans (with 0.01 $HgCl_2$ solution) were soaked in water for a dayat 20°C., placed in the sterilized container with holes at bottom, and then cov-

ered with gauze, then allowed to germinate at room temperature (20−23°C.) in dark place.[3],[6],[16] Watering wasdone four times a day.

After the eight day of cultivation leaves apeared and after the tenth day of germination radicles grew remarkable. They were changed to reddish brown so that the grenbean sprouts could be no longer edible.

iii) Determination of the Total Quantity of Flavin Compounds

To estimate of the fluorescence of flavins separately from those of similar fluorescent substances in the living body, the procedure of converting flavins to lumiflavin is of excellent one.[17],[18] In the living body tissues, the fluorescent substances soluble in chloroform were neglible amount.

The variation of flavin contents in greenbeans during its germination was determined by means of the Yagi's lumiflavin fluorescence method.[11],[20]

The author has estimated the content of flavin in each part of sprout, divided them into two groups, cotyledon group=cotyledon and leaves, hypocotyl group=hypocotyl and radicles.

The weighed germinating greenbeans(a. gr.) were microhomogenized by Potter Microhomogenizer,[21] the homogenate transfered into 100 ml. messflask and the solution was extracted in water-bath(80°C) for just 15 minutes.[22] After cooling it was diluted with distilled water up to 100 ml.(b ml.), then thoroughly mixed. After a portion of the mixture was filtered, the upper layer free from protein was taken for the estimation of flavin: Five milliliter of the upper layer was placed in a test-tube (1.5cm. ×20cm.), 5.0ml. of 1% acetic acid solution and 5.0 ml. of chloroform were added successively. The tube was shaken in cold water, put in dark place for 3 hourse, then 2.0 ml. of chloroform layer was plac d, in the tube A. Two milliliter of a riboflavin standard solution (200 γ%) was also placed in other tube B, and then followed by addition of 2.0 ml. of N-NaOH solution to each tube. Those were subjected to alkali photolysis for 40 minutes.[23] Upon irradiation the flavin compounds were converted into lumiflavin. The irraiated solution in tube A was acidified with 0.2 ml. of glacial acetic acid, two drops of 5% $KMnO_4$ solution added, subsquently with 3% H_2O for removal of interfering non-specidic fluorescent substance to the method of Vitamin Assay. The tube B was also added with 0.2 ml. of glacial acetic acid and water of equal volume of 5% $KMnO_4$ solution and H_2O.

Lumiflavin produced from flavins was extracted with 4.0 ml. of choroform by shaking the tube well in cold water. Two milliliter of chloroform layer was taken in a colourimetric tube, and 0.3 ml. of water was added to it to prevent its evaporation. The intensity of the fluorescence of the chloroform layer was measured by the ultraviolet lamp (220V., 600W.) Calculation was carried out according to the following formula.

$$F=f\times\frac{2b}{a}(r/g)$$

$$F=f\times\frac{2b}{n}(r/g)$$

F: total flavin
a: sample(gr.)
b: messflask (100 ml.)
f: ml. of lumflavin used
n: number of grain

iv) Fractional Determination of Flavin Compounds

Various methods have been offered for the fractional estimation of flavin. In the present study, a paper-chromatoagphic procedure was described by Yagi for this purpose.[25],[26]

To the warm water extract[22] and solution deproteinized with ammoniumsulfate 2.0 ml. of phenol saturated with water was added and this mixture was vigorously shaken, and centrifuged. The flavins were shifted into phenol layer. After the phenol later was removed by a pipette, the residue was again extracted with phenol. To the combined phenol solution 0.5 ml. of redistilled water was added and the mixture was stirred, Ten to twenty milliliters of ether was added to it. The mixture was stoppered, vigorously shaken for one minute in cold

water, then centrifuged. The water layer was separated at the bottom. The phenol-ether layer was discarded and again 5.0 ml. of ether was added to the water layer. The mixture was also stirred and centrifuged. Most of the flavin was thus concentrated in the water layer. The aqueous solution of the flavin compounds was spotted on a filter paper (Whatman No. 1, 20×45cm.) in a dark room. This paper was submitted to one dimensional paper-chromatography in dark place for 15~20 hours: The solvent system was composed of n-buthanol: acetic acid: water (4: 1: 5) (the upper layer) as the mobile phase.[27]

After drying it in a dark room, the fluorescent chromatograms of flavins on paper strip were identified as little as 0.01 ugm. or flavin by ultraviolet light and then washed with ether. A part of the paper strip containing each flavin was cut off 5mm×5mm. in size. It was extracted with hot water (80°C). The extracts were subjected to the determination of FAD, FMN and FR by the lumiflavin fluorescence method as described above. Rf values of the chromatograms are shown in Table 1.

flavins	Rf values
FAD	0.03-0.045
FMEMN	0.09-0.10
FR	0.27-0.30

Table 1. Rf values of flavin compounds

The calculation was as following formula.

FAD : FMN : FR a:b:c

$$\text{FAD} = \text{total flavin (r/grain)} \times \frac{a}{a+b+c}$$

$$\text{FMN} = \text{total flavin (r/grain)} \times \frac{b}{a+b+c}$$

$$\text{FR} = \text{total flavin (r/grain)} \times \frac{c}{a+b+c}$$

Results and Discussion

i) Growth and Total Quantity of Flavin Compounds

The daily changes in the total quantity of flavin compounds of cotyledon and hypocotyl in the greenbean germination as well as their growth for nine days are shown in Fig. 1 and Table 2, 3 and 4.

days of germination	lenght of hypocotyl (mm)	moisture(%)	r%	dry base
1	4-8	61.10	50	128.53
2	10-15	80.08	57	286.14
3	25-30	92.37	60	786.36
4	45-60	93.10	68	985.50
5	60-90	94.43	61	1,095.15
6	100-120	94.59	77	1,423.29
7	120-130	94.62	92	1,710.03
8	130-150	95.12	98	2,008.19
9	150-160	96.10	64	1,641.02

Table 2. The total quantity of flavin compounds in hypocotyl during greenbean germination

days of germination	moisture (%)	r%	dry base
1	59.80	49	121.89
2	62.29	80	212.15
3	65.55	120	348.33

4	72.45	150	544.46
5	75.25	156	630.30
6	79.00	158	752.38
7	80.00	179	895.00
8	83.20	195	1,160.71
9	85.30	138	938.77

Table 3. The total quantity of flavin compounds in cotyledon during greenbean germination

days of germination	cotyledon (γ)	hypocotyl(γ)	total(γ)
1	0.18	0.02	0.20
2	0.19	0.03	0.22
3	0.25	0.08	0.33
4	0.30	0.14	0.44
5	0.31	0.17	0.48
6	0.35	0.22	0.57
7	0.38	0.25	0.62
8	0.41	0.30	0.71
9	0.39	0.24	0.63

Table 4. Formation of flavin compounds in each of greenbean grain during its germination.

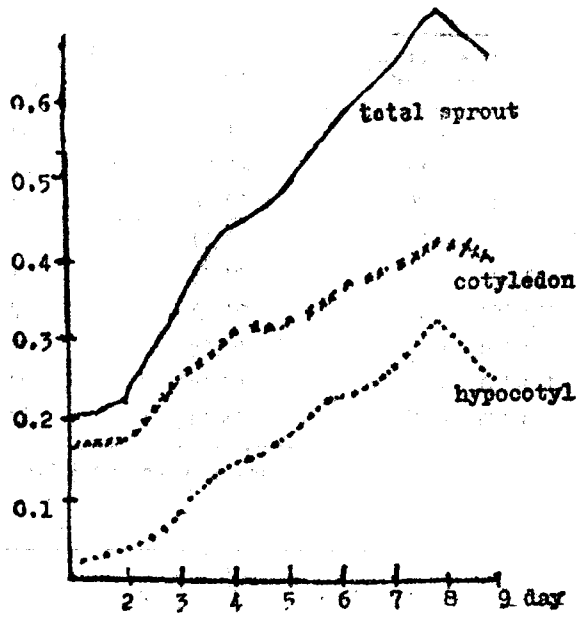

Fig. 1. Formation of riboflavin in each of greenbean grain during its germination

Generally, the total quantity of the flavin compounds of greenbean sprouts was increased with the growth of the sprouts and reached to the amount of three times in cotyledon and hypocotyl as much as the content in original greenbeans. After the appearance of the first leaves, their contents were decreased slightly. In view of sectional distribution of flavin described above, the total quantity of flavin compounds in cotyledon appeared to be higher than that of hypocotyl. It may be therefore suggested that the formation of flavin was mostly performed in cotyledon and then transferred to lower part of the sprouts (hypocotyl). [28]

ii) Ratio of three forms of flavin.

The quantity of three forms of flavin in cotyledon and hypocotyl in the greenbean germination for nine days are given in Table 5,6 and Fig. 2,3.

There were no remarkable difference in the contents of FNM and FR in the cotyledon but the major part of the total quantity of flavin compounds in the sprouts comprised FAD and therfore the changes in the amount must follow the pattern of FAD change therein. [29][30] The variation of both FMN and FR contents were insignificant in cotyledon as shown in the Fig. 2.

On the other hand, the FAD of hypocotyl comprised the major part of ester forms until the 6th day of the germination but after this period their contents were decreased suddenly. Although the quantity of FMN was reached maximun value on the 8th day of germination, the quantity of FR was increased gradually during its germination. At the last stage of germination the content of FR was higher than those of FAD and FMN. The variation of FAD, FMN and FR contents were natable in the hypocotyl but less so in the cotyledon.

Even though the ocurrence of riboflavin and component of several enzymes concerned with respiration is well known, little is known about the mechanisms of biosynthesis of flavin nucleotides in plants. From these series of results, it may be concluded that free riboflavin can be converted by enzymes to FMN and FAD with a high concentration riboflavin. It may also be considered that flavokinase activi ty in greenbean sprouts increased considerable during its germination. [31] FMN was synthesized by flavokinase by means of irreversible reaction with APT, ADP. [14],[12],[33]

The synthesis of FAD from riboflavin may proceed through the initial phosphorylation of riboflavin [12],[14],[33] and subsequent interaction of FMN and ATP to form FAD by FAD synthesizing enzymes in a reversible reaction. [13],[34]

A metallic ion is also required for full activity. Zn^{++}, Mg^{++} are effective in the FAD-sy nthesizing enzymes system. [32]

This scheme is shown as follow.

$$FR \xrightarrow[ATP,\ ADP]{\text{flavokinase}} FMN$$

$$\leftarrow \xrightarrow[ATP,\ ADP,\ Mg^{++},\ Zn^{++}]{\text{FAD-synthesizing enzymess}} FAD$$

Therefore, this type of phosphorylation of riboflavin may be expected to be of biochemical significance.

days of germinntion	total quantity of flavin compounds in cotyledon (γ)	FAD(γ)	FMN(γ)	FR(γ)
1	0.18	0.10	0.05	0.03
2	0.19	0.10	0.06	0.03
3	0.25	0.18	0.05	0.02
4	0.30	0.20	0.09	0.01
5	0.31	0.21	0.08	0.02

6	0.35	0.21	0.09	0.05
7	0.38	0.28	0.03	0.02
8	0.41	0.33	0.05	0.03
9	0.39	0.27	0.04	0.08

Table 5. Variation of FAD, FMN and FR in cotyledon

days of germination	total quantity of flavin compounds in hypocotyl(γ)	FAD(γ)	FMN(γ)	FR(γ)
1	0.02			
2	0.03			
3	0.08			
4	0.14	0.08	0.04	0.02
5	0.17	0.12	0.02	0.03
6	0.22	0.16	0.04	0.05
7	0.25	0.08	0.12	0.05
8	0.30	0.06	0.17	0.07
9	0.24	0.03	0.06	0.15

Table 6. Variation of FAD, FMN and FR in hypocotyl

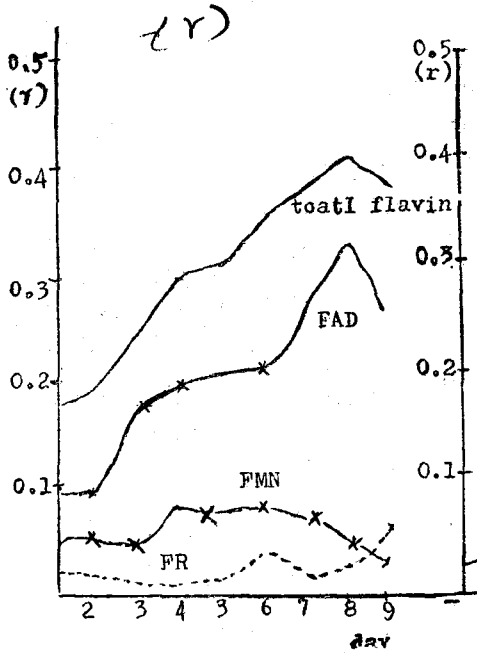

Fig. 2. Variation of FAD, FMN and FR in cotyledon

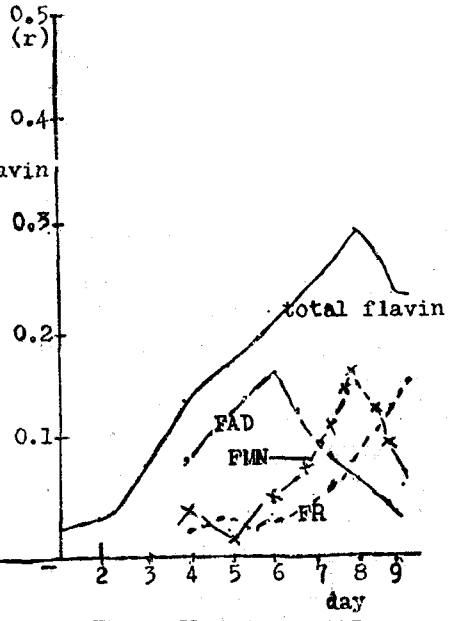

Fig. 3. Variation of FAD, FMN and FR in hypocotyl

總 括

綠豆發芽에 따르는 flavin化合物(FAD, FMN, FR.)의 含量變化를 八木氏의 lumiflavin比螢光法으로 測定하여 다음의 結果를 얻었다.

1. 發芽와 더부러 flavin化合物의 總量은 幼葉出現(8日째)까지 直線的으로 增加되나 그後 그의 含量은 若干 減少되는 傾向을 보였다.
2. flavin化合物은 free riboflavin으로부터 生成됨

을 알았다.

3. 子葉部의 flavin은 FAD가 그의 大部分을 차지하였으며 幼莖部에서는 FAD, FMN, FR含量이 顯著한 差異를 나타냈다.

Summary

The daily variation in the total quantity of flavin compounds (FAD, FMN, FR) in greenbean during its germination were measured by Yagi's methods.

The results obtained were as follow.

1. The total quantity of flavin compounds of the greenbean sprouts was increased with growth of sprouts until the 8th days. After this period its contents were decreased slightly.
2. The flavin compounds of greenbean sprouts were comfirmed to be synthesized as free riboflavin during its germination.
3. FAD was the major pat of total flavin compounds in cotyledon but there was significant variation in the contents of FAD, FMN and FR in hypocotyl.

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