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Investigations of Vicilin and Legumin

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In the seeds of different species of the family *Leguminosae* there occur two well-defined globulin components, legumin and vicilin, which were first isolated from seeds of peas, *Pisum sativum*, by Osborne [1]. He made many analyses with high accuracy on dried preparations of the two components. It is, however, impossible to decide if his preparations were monodisperse, and if the proteins had been denatured. However, with the ultracentrifuge one may investigate the homogeneity of protein solutions. Legumin has been investigated in the ultracentrifuge by Sjögren and Svedberg [2] and they determined the sedimentation constant of legumin to be $s_{20} = 13.1$ S (the value reduced to water basis) [3]. During the last year [4] it was shown that legumin and vicilin occur practically throughout the family *Leguminosae*. Methods of separation of the two globulin components have been developed which are based on the fact that legumin is precipitated at pH 4.7 and vicilin is not [4]. With this method it is possible to separate the components from each other completely, and molecular weight determinations have given the values $M = 186\,000$ for vicilin and $M = 331\,000$ for legumin [4]. Thus the molecular weight of legumin is about twice that of vicilin.

CHEMICAL DIFFERENCES BETWEEN LEGUMIN AND VICILIN

It is not yet clearly settled if vicilin and legumin are two different globulins. Vicilin could be a dissociation product of legumin, or legumin could be formed from vicilin. This question will be taken up in this paper. Osborne and Campbell [5] found small but distinct differences in the chemical composition, especially in the sulphur content, and the difference in solubility also seems to confirm the hypothesis that we are dealing with two substances which are not very closely related. In this paper some quantitative determinations of tyrosine and tryptophan will be reported. The method of determination was

that developed by Goodwin and Morton [6], and only a brief account of the method will be given here. Only a few amino acids, tyrosine, tryptophan and phenylalanine, show absorption in the region 250—320 mμ. The absorption of tyrosine and tryptophan is much stronger than that of phenylalanine, and using 0.1 *N* NaOH as solvent the maximum of the absorption of phenylalanine occurs near 258.5 mμ with a low molecular extinction. Goodwin and Morton have selected wave-lengths at which phenylalanine makes no contribution. The extinction curves of tyrosine and tryptophan intersect at 257.15 and 294.4 mμ. By using the intersecting point 294.4 mμ it is possible to determine tyrosine and tryptophan with a rather high accuracy even if phenylalanine is present.

Experimental

About 10 mg of substance was dissolved in 10.0 ml 0.1 *N* NaOH. The absorption of this solution was determined in a Beckmann photoelectric spectrophotometer at 280, 294.4, 340 and 370 mμ.

Method of calculation

The observed extinction coefficients at 280 and 294.4 mμ are used in the determinations of tyrosine and tryptophan. These values must, however, be corrected for the irrelevant absorption, and for this the extinction coefficients at 340 and 370 mμ are used. The molecular extinction coefficients for tyrosine are $E_{280} = 1576$ and $E_{294.4} = 2375$; for tryptophan $E_{280} = 5225$ and $E_{294.4} = 2375$. If one considers the irrevelant absorption as a straight line in the wave-length range 280—370 mμ, the correction factor for an observed extinction coefficient E^{obs} is

$$\varepsilon = E_{370} + \frac{E_{340} - E_{370}}{370 - 340} (370 - \lambda) \tag{1}$$

$\lambda = 280$ and 294.4 mμ. Then

$$E^{corr} = E^{obs} - \varepsilon \tag{2}$$

If the concentration of tyrosine is y g · mol/l and that of tryptophan $(x—y)$ g · mol/l, we have:

$$x = \frac{E^{corr}_{294.4}}{2375} \tag{3}$$

and

$$E^{corr}_{280} = y \cdot 1576 + (x—y)\ 5225 \tag{4}$$

(3) and (4) give

$$y = \frac{5225 \cdot E_{294.4}^{corr} - 2375 \cdot E_{280}^{corr}}{2375\ (5225 - 1576)} \qquad (5)$$

The molecular weights are for tryptophan 204.2 and for tyrosine 181.2. If A grams of protein is dissolved in 10.0 ml 0.1 *N* NaOH, we have $\frac{(x-y) \cdot 204.2}{A}$ % tryptophan and $\frac{y \cdot 181.2}{A}$ % tyrosine.

The results of the measurements are found in Table 1. The measurements were made on dried preparations of legumin and vicilin from peas. Some of the preparations were over one year old, and some of them were newly prepared.

Table 1. Determination of the tyrosine and tryptophan content of vicilin and legumin by measurement of absorption in the ultra-violet. The values of E are observed values.

Preparation	Substance mg	E_{280}	$E_{294.4}$	E_{340}	E_{370}	Tyrosine %	Tryptophan %
Vicilin							
10	10.0	0.432	0.560	0.020	0.013	3.88	0.29
11	10.7	0.432	0.570	0.022	0.016	3.74	0.24
22	10.1	0.424	0.550	0.011	0.007	3.81	0.30
27	10.3	0.430	0.560	0.019	0.013	3.79	0.27
29	9.8	0.409	0.538	0.012	0.007	3.85	0.26
					Average:	3.81	0.27
Legumin							
8	10.0	0.658	0.658	0.027	0.021	3.84	1.19
9	10.6	0.762	0.743	0.029	0.022	3.98	1.39
10	10.1	0.660	0.662	0.040	0.032	3.81	1.16
11	10.6	0.762	0.748	0.048	0.034	3.97	1.30
22	10.4	0.695	0.698	0.045	0.032	3.85	1.15
					Average:	3.89	1.24

It is seen from the values in Table 1 that vicilin and legumin contain about the same amount of tyrosine, 3.81 and 3.89 %, which values are considerably higher than those obtained by Osborne and coworkers [7,8]. With the method described here, the tyrosine content can be determined with satisfactory accuracy; the maximum deviation from the average is 1.8 % for vicilin and 2.3 % for legumin. In the tryptophan determinations the deviations are considerably higher, 11 % for vicilin and 12 % for legumin. According to these investigations vicilin contains 0.27 % tryptophan and legumin 1.24 %, which values show that vicilin and legumin from peas differ in

their chemical composition. Therefore vicilin and legumin cannot be very closely related. It must be stated that the analyses described here were made on fractions of vicilin and legumin which were shown to be homogeneous in the ultracentrifuge. It is interesting to compare these investigations with the results obtained by Osborne and Campbell [5]. They investigated legumin from different species (pea, lentil, horse bean, vetch) and found that legumin preparations from the different species had the same chemical composition. The same held for vicilin from the different species, but distinct differences in chemical composition between legumin and vicilin could be shown. The following table is taken from Osborne and Campbell.

Table 2. The composition of legumin and vicilin according to Osborne and Campbell.

	Legumin					Vicilin			
	Pea	Lentil	Horse bean	Vetch	Average	Pea	Lentil	Horse bean	Average
Carbon	51.74	51.73	51.72	51.69	51.72	52.36	52.13	52.38	52.29
Hydrogen	6.90	6.89	7.01	6.99	6.95	7.03	7.02	7.04	7.03
Nitrogen	18.04	18.06	18.06	18.02	18.04	17.40	17.38	17.52	17.43
Sulphur	0.42	0.40	0.39	0.43	0.41	0.18	0.17	0.15	0.17
Oxygen	22.90	22.92	22.82	22.87	22.88	23.03	23.30	22.91	23.08

From the experiments described above the following conclusions can be drawn. The differences in tyrosine and tryptophan content of vicilin and legumin from peas show that we are dealing with two well defined globulin components which are not very closely related. The results of Osborne and Campbell [5] show that legumin and vicilin from other species in the *Leguminosae* are identical with legumin and vicilin from peas, in respect to the chemical composition. Ultracentrifugal experiments [4] showed that all leguminosae plants investigated (about 30 species) contain vicilin and legumin.

THE ISOLATION OF LEGUMIN

The difference in tryptophan content in legumin and vicilin was assumed to be great enough for quantitative determinations of the two globulin components when they occurred in the same solution. This proved to be true under certain conditions. When pure preparations of legumin and vicilin were dissolved in 0.1 *N* NaOH in known concentrations, it was possible to determine the tryptophan content of such a solution, and with a simple mathematical calculation the amounts of legumin and vicilin could be determined from this value with satisfactory accuracy. When this method was tried on some pre-

parations in which legumin and vicilin had not yet been separated by isoelectric precipitation, very high tryptophan values were obtained. This must depend on a third substance with high tryptophan content. Experiments were carried out to isolate this third component. Therefore the tyrosine and tryptophan content was determined on preparations of pea globulins containing both vicilin and legumin, which had been precipitated by dialysis against water. In these investigations the determinations of the absorption were made in the following way. The wet precipitate was dissolved in 0.1 *N* NaOH and the solution investigated in the Beckmann photometer. The supernatants were similarly treated by adding NaOH to 0.1 *N*. In this way it was impossible to calculate the percentage content of tyrosine and tryptophan. With the use of equations (1)—(5) the fraction

$$F = \frac{y}{x—y}$$

can be obtained which is the molar ratio $C_{\text{tyrosine}}/C_{\text{tryptophan}}$ of the solution. This fraction is for vicilin $F = 16.1$ (average value from 15 determinations) and for legumin $F = 3.6$ (from 19 determinations). In solutions containing a mixture of these two globulin components, one should have

$$3.6 < F < 16.1$$

As was said above, preparations in which legumin and vicilin had not yet been separated abnormally high tryptophan values were obtained, *i. e.* $F < 3.6$. The separation of the two components was carried out by repeated dialysis against buffer solutions of pH 4.7 and water according to the scheme below. After each dialysis against water both the precipitate and supernatant were investigated in the Beckmann photometer in the manner described above. The values are given in Table 3. The preparation containing both legumin and vicilin (fraction 9 in the preparation scheme) had $F = 1.6$, which is very low.

As is seen from Table 3 most of the substance with high tryptophan content ($F < 1$) remained in solution after dialysis against water, but part of it was precipitated. The F values for vicilin and legumin increase after each dialysis, but it is impossible to get still higher values if the dialyses are repeated more times than in the preparation scheme described here, *i. e.* the component with high tryptophan content is completely removed after the second dialysis against water (fractions 17 and 23 in the preparation scheme).

Preparation scheme for the isolation of vicilin and legumin from peas.

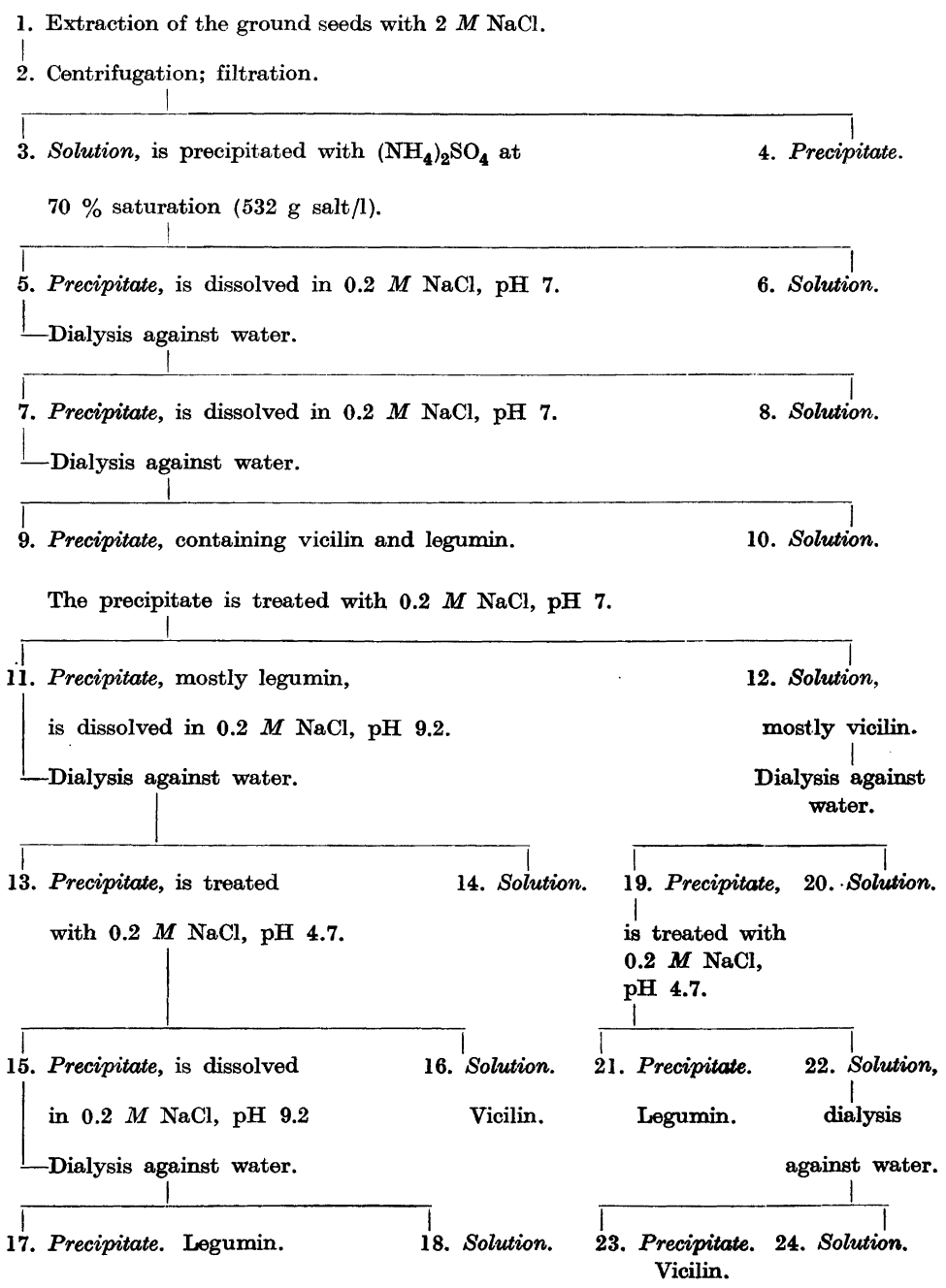

Table 3. Determination of $F = C_{tyrosine}/C_{tryptophan}$ *in precipitates and supernatants after dialysis of vicilin and legumin against water.*

	Fraction in the preparation scheme	Precipitate F	Solution (supernatant) F
Vicilin			
Before 1st dialysis	(12)	—	3.5
After 1st dialysis	(19) (20)	12	0.5
After 2nd dialysis	(23) (24)	16	—
Legumin			
Before 1st dialysis	(11)	1.2	—
After 1st dialysis	(13) (14)	3.6	0.3
After 2nd dialysis	(17) (18)	3.9	0.8

This component with high tryptophan content ($F < 1$) has been isolated in larger amounts in the form of a white amorphous powder by drying in vacuum the supernatants from dialyses against water. Ultracentrifugal analyses showed that it is a low-molecular substance, and part of it goes through the sac during dialysis. It is probably identical with legumelin, first obtained by Osborne [1], who defined legumelin as a substance, which is partly precipitated by dialysis and this fact has been confirmed in the experiments described above.

A SEROLOGICAL COMPARISON BETWEEN LEGUMIN AND VICILIN

In order to decide whether legumin and vicilin have similar serological properties in spite of differences in chemical constitution and molecular weight, some experiments with intravenous injections of rabbits have been carried out [9]. One rabbit was given intravenous injections with a solution of legumin and another rabbit was injected with a solution of vicilin. The two globulin components had been isolated from seeds of *Pisum sativum*. As far as could be seen from ultracentrifugal experiments the two components were entirely separated. When the immunization was finished, precipitin reactions were carried out on the immune sera obtained according to Table 4. Precipitin reactions on the above sera were also carried out with globulin solutions from seeds from other species in the family *Leguminosae*. These solutions contained both legumin and vicilin [4].

At the experiment described in this paper, each rabbit was injected every seven days, with 0.1 % solutions of legumin respectively vicilin. The total quantity injected in each rabbit was 0.015 g of the protein. In the precipitin

reactions 0.25 ml serum was mixed with 0.25 ml of 0.5 % protein solution in a test tube at 20° C. Experiments were also carried out at lower concentrations but the same results were obtained as those described here. Altogether six experiments have been made, but in no case any deviations from the results in Table 4 have been observed.

Table 4. Precipitin reactions of globulin solutions from various species in the Leguminosae. The sera used were obtained by injecting rabbits with solutions of vicilin and legumin from peas.

Protein and species	Legumin serum Reaction after 2 h	Legumin serum Reaction after 24 h	Vicilin serum Reaction after 2 h	Vicilin serum Reaction after 24 h
Legumin				
Pisum sativum	2 +	3 +	2 +	3 +
Vicilin				
Pisum sativum	—	2 +	—	2 +
Legumin + Vicilin				
Glycine Soja	—	+	—	+
Lathyrus Clymenum	+	2 +	+	2 +
Lathyrus odoratus	2 +	3 +	2 +	3 +
Lathyrus silvestris	+	2 +	+	2 +
Lupinus albus	—	2 +	—	2 +
Lupinus angustifolius	+	2 +	+	2 +
Medicago sativa	—	+	—	+
Vicia faba	—	2 +	—	2 +
Vicia sativa	+	2 +	+	2 +

As can be seen from Table 4 all the globulin solutions from different species of the *Leguminosae* gave precipitin reactions with both sera from pea globulins called legumin serum and vicilin serum in the table. This shows that the seed globulins from the different species in this family are immunologically very closely related. It may also be mentioned that α- and γ-globulin from barley and wheat [10, 11] gave no reactions with the sera used above. As is seen from Table 4 vicilin gave precipitin reaction with legumin serum, and legumin with vicilin serum. In spite of differences in chemical composition vicilin and legumin are immunologically related. The cross reactions of the pea globulins may of course depend upon inhomogeneity of the vicilin and legumin preparations, not detectable in the ultracentrifuge.

SUMMARY

1. The tyrosine and tryptophan content of the globulins vicilin and legumin from seeds of *Pisum sativum* has been determined by measurement of absorption in the ultra-violet.

2. Vicilin contains 3.81 % tyrosine and 0.27 % tryptophan, legumin contains 3.89 % tyrosine and 1.24 % tryptophan.

3. From the tryptophan content it is seen that vicilin and legumin differ in chemical composition, as was also shown by Osborne and Campbell by sulphur analyses. Thus vicilin and legumin are two well defined globulin components. It is not very probable that one of these components can be formed from the other by dissociation or association. It has previously been shown in the ultracentrifuge that vicilin and legumin occur in the seeds of most species investigated in the *Leguminosae*.

4. A low-molecular substance with high tryptophan content has been isolated. This substance may be legumelin.

5. A serological experiment with vicilin and legumin from seeds of 10 different species in the family *Leguminosae* showed that the globulin solutions from these species are immunologically very closely related.

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