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An Electrophoretic Investigation of Vicilin and Legumin from Seeds of Peas

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Previous investigations at this institute have shown that the seeds of pea, *Pisum sativum*, contain two globulin components, vicilin and legumin [1] which are well-defined by ultracentrifugation. These components have been separated by different methods, for example precipitation at low temperature or fractionated precipitation with ammonium sulphate. The best method so far for the separation of the two components is iso-electric precipitation of legumin at pH 4.7. By dialysis of a solution containing both vicilin and legumin in a cellophane bag against a buffer at pH 4.7 legumin precipitates and vicilin stays in the supernatant solution. The preparation scheme for the isolation of vicilin and legumin from peas has been described in a previous paper which also deals with the differences between the ultra violet absorption of the two components [2]. By measurement of light absorption in the ultra violet region of the components in 0.1 N NaOH it is possible to calculate the tyrosine and tryptophan contents. Legumin and vicilin contain the same amount of tyrosine, but legumin contains five times as much tryptophan as does vicilin. Thus vicilin and legumin are different in their chemical composition. The molecular weights are also different; determinations have given the values $M = 186\,000$ for vicilin and $M = 331\,000$ for legumin [1]. In this paper the electrophoretic properties of the two components will be discussed. In a recent paper, published when most of the work described here was already completed, Wetter and McCalla have dealt with the same problem [3]. However, they have not investigated the isolated components. A comparison with their results will be made later on in this paper.

EXPERIMENTAL

Ground seeds from peas are extracted over night at + 4° C with 1 *M* NaCl buffered to pH 7. After centrifugation and filtration of the extract the proteins (vicilin, legumin and albumin) are precipitated with $(NH_4)_2SO_4$ to 70 % saturation. The resulting precipitate is dissolved in 0.2 *M* NaCl, pH 7, and the solution is then dialysed in a cellophane bag against water. (One night against running tap water, and then distilled water.) When the salt concentration is lowered by dialysis, the globulins precipitate. The precipitate is dissolved in 0.2 *M* NaCl, pH 7, and again dialysed against water. After this second dialysis the globulins, which are now free from albumins, are dissolved in unbuffered 0.2 *M* NaCl, and this solution is dialysed against a buffer solution of pH 4.7 (0.2 *M* NaCl). During this dialysis legumin is iso-electrically precipitated, and the vicilin remains in solution in the supernate. By repeated dialysis at pH 4.7, vicilin and legumin can be quantitatively separated, as indicated by the ultracentrifuge. The resulting vicilin and legumin preparations are finally precipitated in a salt free state by dialysis against distilled water, and freeze-dried in vacuum. They are obtained in the form of a white powder, readily soluble in 0.2 *M* NaCl at pH 7.

The same freeze-dried preparations of vicilin and legumin have been used in all the experiments described here. Comparisons have been made with other highly purified preparations but no differences have been observed. In other crude preparations, however, it was possible to show incomplete separation as will be mentioned later.

INVESTIGATIONS OF VICILIN

Solutions were prepared containing about 0.8 % dried vicilin in 0.2 *M* NaCl, buffered to different pH values ($\mu = 0.25$). The solution (18 ml), was dialysed against 2000 ml of the buffer for 48 hours at + 4° C, and then analyzed at + 5° C. in the Tiselius' electrophoresis apparatus [4] with the modifications indicated by Svensson [5]. Solutions of vicilin and especially legumin are very difficult to investigate at lower temperatures because they precipitate when the temperature is lowered. Legumin must be investigated at + 15° C. It seems, however, as if this sensitivity to low temperature is greater when the components are purified. Perhaps some stabilizing colloid is removed by purification.

A typical electrophoretic diagram of vicilin is shown in Fig. 1. One single peak is obtained. Thus vicilin, which is homogeneous by ultracentrifugation, is also homogeneous by electrophoresis. In investigations of other vicilin preparations which had not been sufficiently purified, a second peak appeared in the diagrams, probably consisting of legumin. Its concentration was about 5—10 % of that of the vicilin. Diagrams similar to Fig. 1 were obtained in the whole pH-interval investigated (pH 3.7—9.3). The results of the measurements are shown in Table 1 and Fig. 2. The isoelectric point for vicilin is determined from Fig. 2 to be pH 5.5.

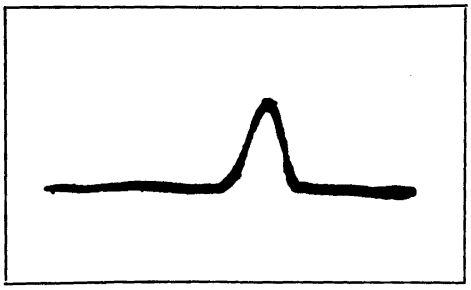

Fig. 1. Electrophoretic diagram of vicilin. pH 4.33. Concentration 0.7 %. Potential 3.01 volts/cm. Migration at + 5° C for 380 min. to the right. The descending peak has migrated 2.0 cm in the apparatus. $\Theta = 70°$. Θ = angle of inclination of the diagonal slit; this equals zero in the vertical position.

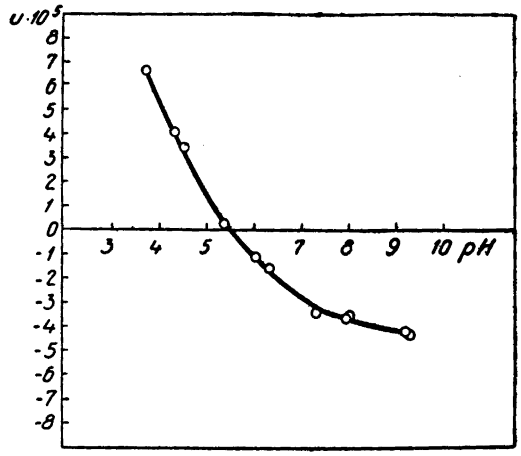

Fig. 2. Electrophoretic mobility of vicilin at different pH values.

INVESTIGATIONS OF LEGUMIN

In order to state whether vicilin and legumin have different electrophoretic properties, the following experiment was carried out. At pH 7.99 a mixture of vicilin and legumin in about the same concentrations (checked by ultracentrifugation) was analyzed in the electrophoresis apparatus at + 5° C. In this case the two components had never been separated from each other by iso-electric precipitation. Two peaks were obtained as can be seen in Fig. 3. The values for the mobilities were 6.95×10^{-5} and 3.79×10^{-5} cm²/volt. sec. The value 3.79×10^{-5} lies on the curve in Fig. 2, and this component must consequently be vicilin. The other component with the mobility 6.95×10^{-5} must be legumin. This experiment proves that vicilin and legumin behave differently in electrophoresis.

After this preliminary experiment some determinations were made on solutions containing purified legumin. As has been stated earlier in this paper, several properties of legumin make electrophoretic investigations rather diffi-

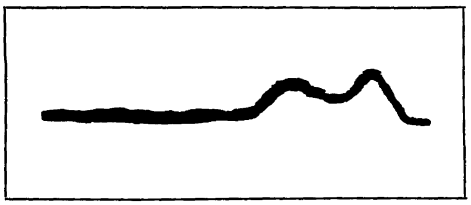

Fig. 3. Electrophoretic diagrams of vicilin and legumin. pH 7.99. Total concentration 0.6 %. Potential 3.65 volts/cm. Migration at + 5° C for 255 min. to the right. The descending peaks have migrated 2.1 cm (vicilin, on the left) and 3.9 cm (legumin, on the right) in the apparatus. $\Theta = 75°$.

Table 1. Electrophoretic mobility of vicilin at + 5° C at different pH Values. All buffer solutions contain 0.2 M NaCl.

Buffer (*M*)	pH	Mobility $\times 10^5$ cm^2/volt sec.
0.008 Na_2CO_3 0.008 $Na_2B_4O_7$	9.28	− 4.32
0.008 Na_2CO_3 0.008 $Na_2B_4O_7$	9.20	− 4.23
0.010 H_3BO_4 0.003 $Na_2B_4O_7$	8.04	− 3.56
0.010 H_3BO_4 0.003 $Na_2B_4O_7$	7.95	− 3.61
0.015 Na_2HPO_4 0.005 NaH_2PO_4	7.31	− 3.45
0.008 Na_2HPO_4 0.026 NaH_2PO_4	6.33	− 1.59
0.006 Na_2HPO_4 0.034 NaH_2PO_4	6.02	− 1.11
0.002 Na_2HPO_4 0.046 NaH_2PO_4	5.37	+ 0.24
0.063 HAc 0.050 NaAc	4.52	+ 3.47
0.100 HAc 0.050 NaAc	4.33	+ 4.04
0.400 HAc 0.050 NaAc	3.72	+ 6.65

cult. Firstly, legumin is iso-electrically precipitated in the pH range 4—6. The precipitation is most complete at pH 4.6—5.6. The solubility behaviour in this range will be described later in this paper. Secondly, legumin is precipitated at low temperature. In the experiments described here the temperature used was + 15° C, at which temperature no precipitation occurs. At this temperature very low current strength (about 8 mA) must be used (the potential

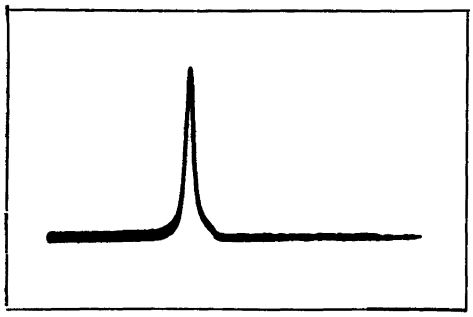

Fig. 4. Electrophoretic diagrams of legumin. pH 3.70. Concentration 1.7 %. Potential 0.99 volts/cm. Migration at + 15° C for 395 min. to the right. The descending peak has migrated 1.4 cm in the apparatus. Θ = 40°.

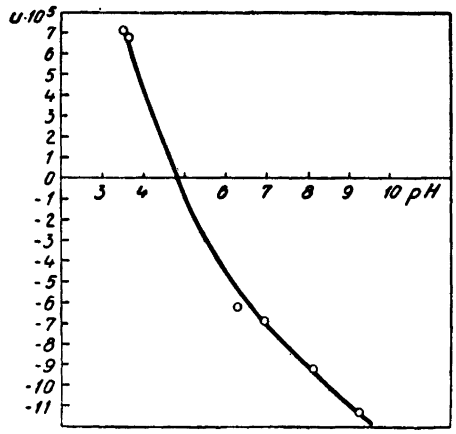

Fig. 5. Electrophoretic mobility of legumin at different pH values.

gradient $F = 0.6—1.0$ volts/cm instead of 2.6—3.6 volts/cm which could be used at + 5° C in the investigations of vicilin).

In electrophoresis of legumin solutions one main peak was obtained as is shown in Fig. 4. Thus legumin is homogeneous by electrophoresis. The results of the measurements are shown in Table 2 and Fig. 5, from which a determination of the isoelectric point of legumin gives the value pH 4.8. The error in this determination is of course rather large.

A COMPARISON BETWEEN THE ELECTROPHORETIC MOBILITIES OF VICILIN AND LEGUMIN AT + 5° C

In order to compare the mobilities for vicilin and legumin the following method was used. As described earlier, an electrophoretic analysis of a solution containing both vicilin and legumin was carried out at pH 7.99 and + 5°C. The mobilities obtained were 3.79×10^{-5} and 6.95×10^{-5} cm²/volt × sec. respectively. The value 3.79×10^{-5} lies on the curve for vicilin at + 5° C (Fig. 2). We can compare the mobilities for legumin obtained above at + 5° C $u = 6.95 \times 10^{-5}$, with the value from the experimental curve in Fig. 5 (which is run at + 15° C). For legumin, at pH 7.99 and + 15° C, was obtained from Fig. 5 $u = 9.05 \times 10^{-5}$. Thus, when the temperature is lowered from 15° C to 5° C, the electrophoretic mobility for legumin is lowered by 23 %. A later experiment showed the corresponding change for vicilin to be 28 %. This is probably largely a result of increased viscosity of the buffer. For water we

Table 2. Electrophoretic mobility of legumin at + 15° C at different pH values. All buffer solutions contain 0.2 M NaCl.

Buffer (*M*)	pH	Mobility × 10^5 cm^2/volt sec
0.008 Na_2CO_3 0.008 $Na_2B_4O_7$	9.27	— 11.3
0.013 H_3BO_3 0.002 $Na_2B_4O_7$	8.15	— 9.20
0.014 Na_2HPO_4 0.010 NaH_2PO_4	6.96	— 6.90
0.008 Na_2HPO_4 0.026 NaH_2PO_4	6.30	— 6.20
0.400 HAc 0.050 NaAc	3.65	+ 6.80
0.450 HAc 0.050 NaAc	3.60	+ 7.12

have the values: $\eta = 1.1404$ centipoises at 15° C and $\eta = 1.5188$ centipoises at 5° C [6]. According to Johnson and Shooter [7] the product $u \times \eta$ is a constant for a certain protein at any temperature. Thus $u_{15°} \times \eta_{15°} = u_{5°} \times \eta_{5°}$, or $\frac{u_{5°}}{u_{15°}} = \frac{\eta_{15°}}{\eta_{5°}}$ should hold in our case. We have $\frac{\eta_{15°}}{\eta_5^\circ} = 0.75$, and from the values above $\frac{u_{5°}}{u_{15°}} = \frac{6.95}{9.05} = 0.77$ for legumin. For vicilin, at pH 7.62, $u_{15°}$ was found to be 4.64×10^{-5} cm^2/volt × sec., and from Fig. 2 we obtain at pH 7.62 and + 5° C $u_{5°} = 3.40 \times 10^{-5}$ cm^2/volt × sec. These values give for vicilin $\frac{u_{5°}}{u_{15°}} = \frac{3.40}{4.64} = 0.73$. Thus $u \times \eta =$ constant holds for both vicilin and legumin and the electrophoretic mobilities of the two components can be compared at the same temperature. The mobility curve for legumin at + 5° can be calculated. After this correction has been made we can conclude that the mobility of legumin is about twice that of vicilin in the pH interval 6.3—9.3 at + 5° C. At pH 3.5, however, the mobility of legumin is lower than that of vicilin.

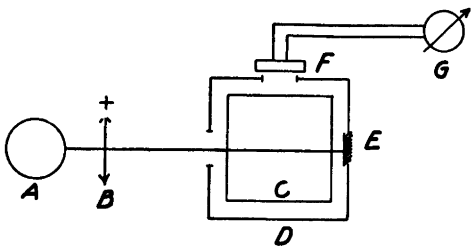

Fig. 6. Nephelometer.
A. Light source (Hg-lamp, "Philora").
B. Positive lens.
C. Glass cell, containing the solution (100 ml)
D. Metal box with windows, containing the cell.
E. Mirror.
F. Photoelectric cell (barrier-layer cell).
G. Reflecting galvanometer. Readings on a circular scale (in mm) with a radius of 90 cm.

DETERMINATION OF THE ISOELECTRIC POINT OF LEGUMIN BY MEANS OF ISO-ELECTRIC PRECIPITATION

From what has been said above, it is difficult to determine the isoelectric point of legumin electrophoretically, because legumin is iso-electrically precipitated. This precipitation was studied by means of a nephelometric method described below, (see Gibb [8] or any other textbook of optical methods). The Tyndall effect of the precipitate was measured at an angle of 90° in an apparatus shown in Fig. 6. There are many sources of error in quantitative nephelometry. Only the two most important will be mentioned: 1) the precipitate is inhomogeneous, *i. e.* the particles are of different sizes. This error can be largely eliminated by measurements on solutions of very low concentration in which case small particles are formed. The wave length of the incident light must consequently be short in order to have high intensity of the scattered light. In these experiments a Hg-lamp has been used. A concentration range where the measured turbidity is a linear function of the amount of substance precipitated must be used. 2) There is an age effect, *i. e.* the small particles form bigger ones. Thus the measurement must be done during the time interval when the age effect has little or no importance.

Determination of the dependence of the turbidity on time

25 ml of a 0.002 % legumin solution in 0.2 *M* NaCl were pipetted into 75 ml 0.2 *M* NaCl, buffered to pH 4.98 with acetate (total legumin concentration 0.0005 %). The solution was then immediately put into the nephelometer, and readings were made periodically as shown in Table 3. The same experiment was performed with a total legumin concentration of 0.002 %. Before and after all measurements in the nephelometer, 100 ml distilled water instead of

Table 3. The dependence of the turbidity on time.

Time after precipitation Minutes	Galvanometer readings $n-n_0$ mm.	
	Legumin conc. 0.0005 %	Legumin conc. 0.002 %
2	88	374
4	95	386
6	98	391
8	100	399
10	101	405
12	101	404
14	102	408
16	102	408
18	103	412
20	103	417
30	106	420
40	106	420
50	105	418
60	104	417

a protein solution were placed in the cell. The corresponding galvanometer reading is called n_0 (usually 0—10 mm) and the readings with protein solutions are called n. In the tables below the difference between the two readings, $n—n_0$ is shown.

It is evident from Table 3 that the measurement must not be made before 20—30 minutes.

Determination of the dependence of the turbidity on the amount of substance precipitated

Solutions containing legumin at different concentrations were iso-electrically precipitated at 3 different pH values. The results of the measurements are shown in Fig. 7. It can be seen that by iso-electric precipitation in the pH-range 4.5—5.6 and the total legumin concentration 0.0005—0.0025% the turbidity is a linear function of the protein concentration.

Determination of the maximum precipitation by iso-electric precipitation of legumin

In order to determine at which pH legumin is most completely precipitated, the following experiment was performed.

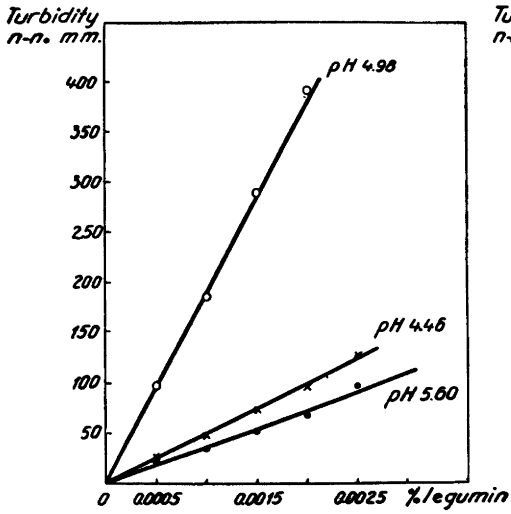

Fig. 7. The turbidity as a function of legumin concentration at different pH values.

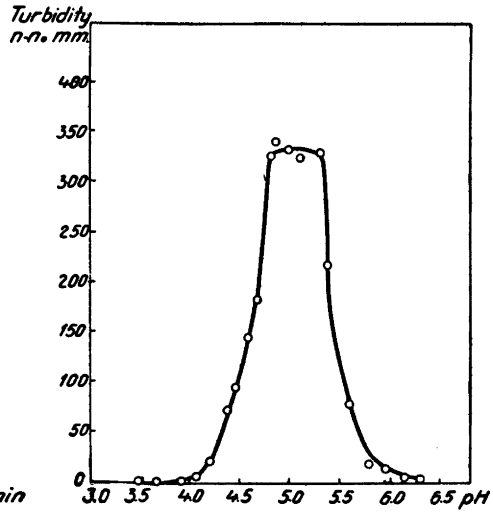

Fig. 8. Isoelectric precipitation of legumin at different pH values.

20 buffer solutions were made, containing acetate or phosphate ($\mu = 0.1$) and covering the pH range pH 3.3—6.3. These buffers also contained 0.2 *M* NaCl (total $\mu = 0.3$). The experiment was carried out in 10 Erlenmeyers flasks (200 ml) as described below. In each Erlenmeyer flask 75 ml of the buffer were pipetted. Every 2 minutes 22 ml 0.009 % legumin solution in 0.2 *M* NaCl were pipetted into one of the flasks, beginning in number one and finishing in number ten. After exactly 30 minutes the turbidity of each solution was measured in the nephelometer. The experiment was then continued in the same way with the 10 remaining buffer solutions. The results are given in Fig. 8.

As can be seen from Fig. 8 legumin is iso-electrically precipitated in a rather narrow pH interval. If we assume that a protein has minimum solubility at its isoelectric point [Rona and Michaelis [9]], we can conclude from this experiment that the isoelectric point of legumin is pH 5.0 $\pm$ 0.2. This result agrees rather well with the value pH 4.8 obtained by electrophoresis.

DISCUSSION

Vicilin and legumin are both practically homogeneous by electrophoresis but their electrophoretic properties are different. They are also different and homogeneous by ultracentrifugation. The author has never observed any dis-

sociation or association reaction by which vicilin is formed from legumin or legumin from vicilin. The differences in chemical composition, for instance the different tryptophan content, and especially the difference in sulfur content, 0.42 % S in legumin and 0.18 % S in vicilin from peas [Osborne and Campbell [10]] is a proof that we are dealing with two well-defined independent globulin components.

Wetter and McCalla [3] believe that it is very improbable that Osborne's methods can give well-defined, homogeneous protein species, and that it should be impossible to separate the different components with simple precipitation methods. The present author considers that with this and previous investigations he has proved this separation to be possible. It is rather simple to isolate vicilin and legumin with precipitation methods, and the resulting protein components are homogeneous by analysis in the ultracentrifuge and electrophoresis apparatus.

SUMMARY

1. Vicilin from peas has been analyzed in the Tiselius' electrophoresis apparatus. In the pH range 3.7—9.3 it is homogeneous.

2. The iso-electric point for vicilin has been determined to be pH 5.5.

3. Legumin from peas also is homogeneous by electrophoresis. Its electrophoretic mobility is higher than that of vicilin in the pH range 6.3—9.3. The iso-electric point for legumin has been determined electrophoretically to be pH 4.8. A nephelometric study of the iso-electric precipitation of legumin gave the value pH 5.0 of the isoelectric point.

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