KININ* ACTIVITY FROM PLANT EXTRACTS

II. PARTIAL PURIFICATION AND FRACTIONATION OF KININS IN APPLE EXTRACT

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Summary

The extraction and partial purification of kinins from *Pyrus malus* cv. Granny Smith fruitlets are described. Tracing progress by means of a biological assay based on cell-division induction in tobacco pith, the kinins, extracted from fruitlets with aqueous ethanol, were partially purified by the following steps: (i) adsorption on a cation-exchange resin and displacement with ammonium hydroxide, (ii) adsorption on carbon and elution with pyridine–ethanol–ammonium hydroxide, and (iii) passage through a polyamide resin. When chromatographed on paper, the kinin activity in the purified extract was resolved into at least four zones, each having independent activity. The kinin of the most active zone was purified 900-fold.

I. INTRODUCTION

The previous paper in this series (Bottomley *et al.* 1963) described a biological assay for plant kinins. This paper sets out the results of purification and fractionation studies on kinins present in an extract of apple fruitlets.

II. MATERIALS AND METHODS

(*a*) *Preparation of Crude Extract*

Apple fruitlets had been found to be a good source of kinins (Bottomley *et al.* 1963) and could be collected in large quantities with unskilled labour. An orchard, largely planted with Granny Smith variety, became available for destruction and this determined the variety used for extraction.

Pyrus malus cv. Granny Smith fruitlets (780 kg) were harvested between 7 and 21 days after pollination. Treatment commenced on most fruitlets within 4 hr of picking. Batches of 20–30 kg of fruitlets, in a stainless steel basket, were immersed three times for periods of 2 min in ethanol at 60°C in a tinned-copper bath. This step was designed to inactivate enzymes. Fruitlets were then drained, cooled, and macerated in a stainless steel swing-hammer mill. The pulp was covered with 70% aqueous ethanol in an enamelled vessel and allowed to stand overnight at room temperature with occasional stirring. The slurry was filtered and the filtrate stored at 4°C. The filter cakes were washed with 70% aqueous ethanol and the washings were used for subsequent extractions. The ethanol used in the initial immersion was used for extraction or was stored for concentration. The total volume of the

* The word "kinin" has also been used for a class of substances which cause constriction of blood vessels and other physiological reactions in animals. These substances, which have been isolated from animal sources, are polypeptides and are unrelated to plant kinins.

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extract was 3000 l. This was concentrated sixfold in a Luwa stainless steel turbofilm evaporator at temperatures ranging from 45 to 60°C. A further sixfold concentration was achieved in a glass climbing-film evaporator at 30–35°C.

This crude concentrate was stored in a stainless steel drum at −15°C. The minimum concentration showing kinin activity was 0·05 g/ml (units defined below), which contained 3·0 g dry matter per litre.

(*b*) *Purification and Fractionation Methods*

(i) *Ion-exchange Resins.*—The cation-exchange resin "Dowex 50" (analytical grade 50, white, 8% cross-linked, 100–200 mesh) was used in the hydrogen form. The anion-exchange resin "Dowex 2" (analytical grade 2, 8% cross-linked, 200–400 mesh) was used in the hydroxyl form.

(ii) *Carbon.*—The carbon used was kindly prepared by Dr. V. A. Garten and Dr. A. B. Whitehead, Chemical Research Laboratories, C.S.I.R.O., by heating "Black Pearl 2" (Godfrey L. Cabot Inc., Boston, Mass.) to 1000°C in oxygen-free nitrogen (Garten and Weiss 1955). This carbon was freed of adsorbed gas by evacuating a slurry in water. Columns of carbon were prepared from this slurry such that their length was at least five times their diameter. Before use, each column was washed with five column volumes of a mixture of equal volumes of ethanol and 2N ammonium hydroxide, and then with water until the effluent was neutral.

(iii) *Polyamide Resin.*—This resin was prepared as follows: 11 kg Du Pont "Zytel" nylon, colour NC10, code 101, was added to 50 l. stirred boiling 90% formic acid and boiling was continued until the nylon dissolved (about 0·5 hr). The solution was then siphoned into a vigorously stirred suspension of 22 kg of "Hyflo-Supercel" (Johns-Mansville Corp., New York) in 200 l. of methanol. The suspension was filtered, washed with methanol, and then with water until free of formic acid. The solid was dried and ground.

(iv) *Paper Chromatography.*—Paper chromatography was carried out both on paper strips and rolls. For the latter a "Chromax" pressure mantle (type 3502) as supplied by LKB Produkter, Stockholm, was used. Either the normal procedure of dissolving the material to be applied to the column in 10 ml of chromatographic solvent was adopted, or the material was dried onto two filter paper disks, which were held at the top of the column by glass beads.

For paper-strip chromatography the extract was streaked onto a line 10 cm from the end of Whatman No. 1 paper 12 by 55 cm. The papers were developed in an appropriate solvent until the front was about 40 cm from the origin. After drying, the developed portion of each paper was divided transversely into strips 0·9 cm wide. These strips were cut into three 4-cm lengths and one length placed into each of three replicate assay tubes to which was added 1 ml of basal medium containing 3-indolylacetic acid (IAA) (Bottomley *et al.* 1963). Two chromatographic solvents were used:

Solvent A: n-butanol–glacial acetic acid–water (4 : 1 : 1 v/v).

Solvent B: methyl ethyl ketone–glacial acetic acid–water (4 : 1 : 1 v/v).

(v) *Expression of Concentration of Plant Extracts.*—For convenience, the concentration of plant extract in a culture medium (Bottomley *et al.* 1963) was expressed in arbitrary units representing the number of grams of fresh apple fruitlets which were extracted for the preparation of 1 ml of medium.

III. Results

(*a*) *Purification of the Crude Concentrate*

(i) *Ethyl Acetate Extraction.*—Routinely, the crude concentrate was continuously extracted with ethyl acetate until no further colour could be removed. This step removed highly coloured material and about one-tenth of the dry matter from the crude concentrate. There was no kinin activity in the extract and no loss of activity from the residue. The minimum concentration showing kinin activity was 2·8 g dry weight per litre.

(ii) *Displacement from Cation-exchange Resin.*—An aqueous solution of ethyl acetate-extracted crude concentrate (vol. 1 l., equivalent to 3·5 kg of fruitlets) was added to a 5 by 70 cm column packed with "Dowex 50". This was washed with 10 l. of water until the effluent was neutral and then the adsorbed material was displaced with 2N ammonium hydroxide. Collection commenced at the appearance of the ammonium hydroxide front and continued until 5 l. were obtained. The ammonia was removed by evaporation under reduced pressure. All of the kinin activity of the original extract was recovered and 0·29 g dry weight per litre was the minimum concentration showing kinin activity.

(iii) *Elution from Carbon.*—In general, the kinins of apple extract were adsorbed onto carbon, but were not readily eluted from most carbon samples investigated. Of several carbon samples tested, only the one described in Section II(*b*)(ii) proved satisfactory.

A water solution of apple extract (equivalent to 4 kg of fruitlets), which had been displaced from "Dowex 50" by ammonium hydroxide, was applied to a column of 400 g carbon. The column was washed with 5 l. water and the effluent was collected. It was then washed with 7 l. ethanol–2N ammonium hydroxide (1 : 1 v/v), and the dark-coloured eluate collected. The final elution was with 1 l. of a mixture of equal volumes of pyridine and the ammoniacal ethanol eluant above. During this elution, the column was heated to 60–70°C by wrapping electrical heating tape around the column.

Kinin activity was detected in the pyridine eluate only and this eluate showed kinin activity at a minimum concentration of 0·028 g dry weight per litre.

(iv) *Passage through Polyamide Resin.*—An extract, purified with "Dowex 50" and carbon and equivalent to 12 kg apples, was applied in water to a 7 by 60 cm column containing 600 g of resin. The column was developed with water and 2 l. of effluent were collected commencing just prior to the issue of coloured material from the column. This step removed some coloured material and achieved a further 1·9-fold purification. In preparations made in this way the greatest dilution which gave perceptible activity was 0·05 g/ml (arbitrary units) and this contained about 15 mg of dry matter per litre.

(v) *Gradient Elution from "Dowex 50".*—Extract equivalent to 1 kg of fruitlets was purified by the "Dowex 50" and carbon steps, dissolved in 10 ml water, and added to a 2 by 20 cm column of "Dowex 50". The column was washed with 400 ml water and these washings showed no kinin activity. The column was then eluted by using the gradient-elution system of Parr (1954). The reservoir contained 6N hydrochloric acid and the mixing flask 0·5N hydrochloric acid. Forty fractions each of 30 ml were collected. The normality of the eluate rose from 0·5 to 4·1. The hydrochloric acid was removed from each fraction by diluting it with six volumes of water and passing through a 1 by 10 cm column of "Dowex 50". After washing free of acid, the kinins were displaced with 2N ammonium hydroxide which was then removed by evaporation.

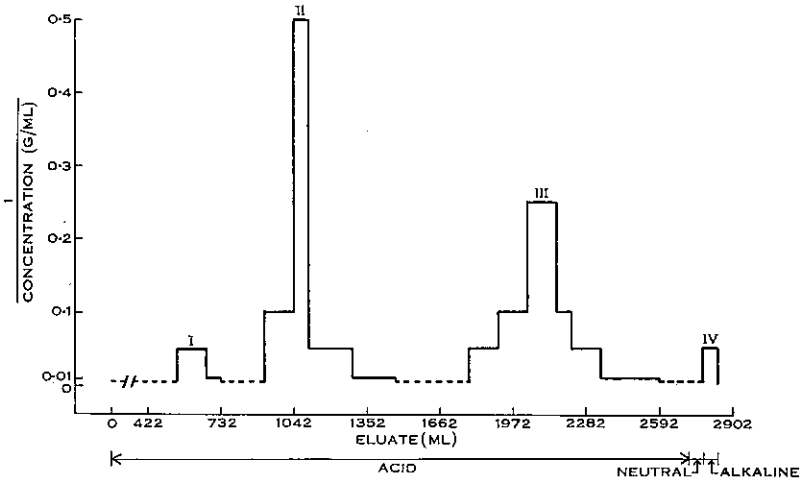

Fig. 1.—Chromatography on a "Chromax" rolled-paper column of partially purified extract of apple fruitlets. Only material soluble in n-butanol–acetic acid–water (4 : 1 : 1 v/v) was applied to the column. The eluting solvents were n-butanol, acetic acid, water (acid), n-butanol saturated with water (neutral), and n-butanol saturated with 2N ammonium hydroxide (alkaline). Fractions of eluate were assayed for cell-division induction in isolated tobacco pith blocks, at concentrations of 0·5, 1·0, 2·5, 5·0, and 25·0 g/ml (arbitrary units—see text). On the vertical axis, the highest dilution producing perceptible cell division is plotted and the broken lines indicate that cell division was not detected at the lowest dilution used.

Although there may have been separation into peaks, the resolution was poor and consequently all the active fractions were combined and used subsequently for separation on a "Chromax" column (see Section III(*b*)(i) below).

(vi) *Adsorption on "Dowex 2".*—Ethyl acetate-extracted crude concentrate, equivalent to 100 g of fruitlets, was added to a 2 by 15 cm column of "Dowex 2". After washing with 150 ml of water the column was eluted with 125 ml of sulphurous acid prepared by saturating water with sulphur dioxide. The sulphur dioxide was removed from the eluate by evaporation to dryness under reduced pressure. The water effluent showed no kinin activity. The sulphurous acid eluate contained about half the applied kinin activity.

(b) *Fractionation by Paper-roll Chromatography*

(i) *Development of Column with Solvent A.*—Extract equivalent to 500 g of apples was purified by the "Dowex 50", carbon, and polyamide steps, and applied to the "Chromax" column in 10 ml of solvent A. The column was developed with solvent A and after an initial fraction of 300 ml, 39 62-ml fractions were collected. The column was then washed with 500 ml of n-butanol saturated with water, followed by 500 ml of n-butanol saturated with 2N ammonium hydroxide.

Each fraction and the washings were evaporated under reduced pressure and assayed over a range of concentrations. The results, plotted in Figure 1, showed three distinct peaks of activity in the first solvent and one in the last.

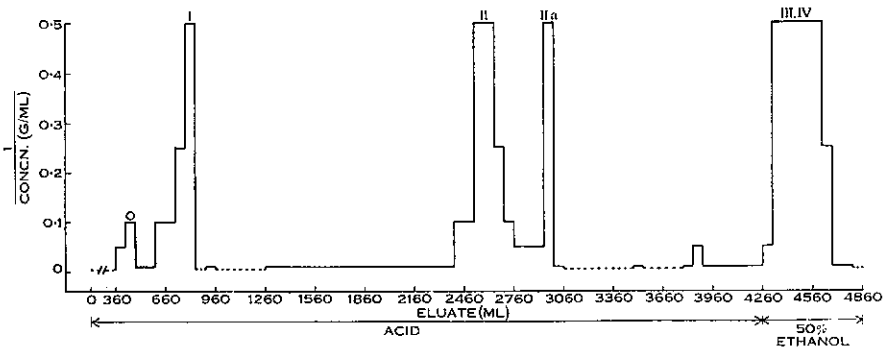

Fig. 2.—Chromatography on a "Chromax" rolled-paper column of partially purified extract of apple fruitlets. The eluting solvents were methyl ethyl ketone–acetic acid–water (4 : 1 : 1 v/v) (acid), and 50% aqueous ethanol. Fractions of eluate were assayed for cell-division induction in isolated tobacco pith blocks as described in Figure 1.

On paper strip chromatograms in solvent B the active peaks had the following R_F values:

Peak	R_F	Peak	R_F
I	0·76	III	0·27–0·34
II	0·37–0·45	IV	0·13–0·23

Dry weight determinations and a dilution-series biological assay showed that peak II kinin was active at 18 mg/l and peak III kinin at 3·5 mg/l. The latter was the most active concentrate prepared and represented on 900-fold concentration over the crude concentrate.

(ii) *Development of Column with Solvent B.*—Extract equivalent to 500 g of fruitlets, purified as in the previous experiment, was applied by drying it onto filter paper. This column was developed successively with one 600-ml and 60 62-ml fractions of solvent B and then 10 60-ml fractions of 50% ethanol. The results are plotted in Figure 2. There were four peaks of activity from the first solvent and one from the second. On paper-strip chromatograms in solvent B the following R_F values were obtained:

Peak	R_F	Peak	R_F
I	0·75–0·84	III–IV	0–0·31
II	0·41–0·54		

No kinin activity was detected when aliquots from peaks 0 and II*a* were rechromatographed.

Peak III, IV kinins were heated in 1N hydrochloric acid at 80°C for 30 min and the solution freed from acid as previously described. A paper-strip chromatogram of this acid-treated kinin in solvent B showed two active areas at R_F 0·06–0·15 and 0·39–0·49, whereas the R_F of the original kinin was 0–0·30. Treatment of peak III, IV kinins with hot 1N sodium hydroxide resulted in almost complete loss of activity.

(*c*) *Attempted Correlation with Ultraviolet Absorption*

The gradient elution step had separated much of the ultraviolet-absorbing material from the kinins. It was therefore possible to attempt to correlate spectrophotometric absorption with kinin activity in the fractions from a "Chromax"

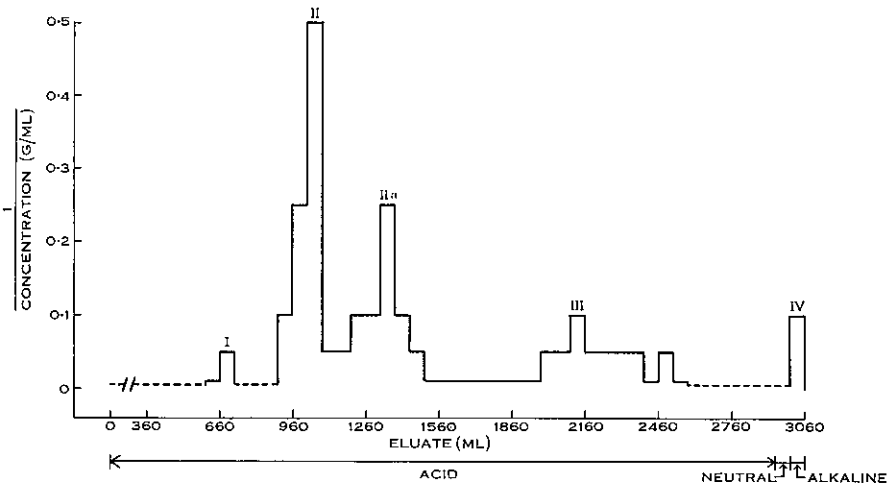

Fig. 3.—Chromatography on a "Chromax" rolled-paper column of apple extract which had been gradient eluted from "Dowex 50"-H^+ with hydrochloric acid. The application of material to the column and its development were as described in Figure 1. Fractions of eluate were assayed for cell-division induction in isolated tobacco pith blocks as described in Figure 1.

column. The combined fractions from gradient elution of "Dowex 50" (see above) were dissolved in 10 ml of solvent A and applied to the "Chromax" column. The column was developed as was the column described in Section III(*b*)(i) above. The results, plotted in Figure 3, showed four peaks of activity in the first solvent and one in the last. However, peaks II and II*a* behaved identically on a paper-strip chromatogram in solvent B.

The absorbance of each fraction was measured between 250 and 350 mμ. Although there were ultraviolet-absorbing materials present, there was no correspondence between absorbance and kinin activity.

IV. Discussion

The kinins, like other growth substances, appear to be active at high dilution as is indicated by the known activity of kinetin and also by our own evidence (see Section III(*b*)(i)) as well as that of others (Miller 1961; Beauchesne 1962). Therefore, despite the large amount of apples collected, the total weight of the kinins present in the extract must be assumed to be small. Because of this, only steps which returned a high yield could be considered suitable for the purification scheme. In addition, in the earlier stages of purification, large weights of material were involved and therefore only procedures suitable for large-scale application were used.

The sole method of detecting activity was the biological assay, which is sensitive to most of the reagents commonly used in purification procedures. Therefore, the complete removal of added reagents was necessary before every assay. For this reason readily volatile solvents were generally preferred.

The kinins of apple fruitlets, like those of most other natural sources investigated, are soluble in water but have negligible solubility in organic solvents. This behaviour contrasts with that of the auxins and gibberellins, which are readily extracted by organic solvents from aqueous extracts and in this way can be purified manyfold. It was therefore necessary to find alternative procedures, which would achieve a large purification in the initial stages.

The kinins extracted from apple fruitlets were adsorbed by the strong cation exchanger "Dowex 50" and were thus separated from much non-ionic and anionic material. The kinins were removed from the resin with ammonium hydroxide, but their activity was detected behind the ammonia front rather than immediately in front of it, as would be expected if ionic forces were responsible for the binding. The strong adsorption, shown by the difficult and slow elution with hydrochloric acid, was thus probably due to non-ionic forces.

The apple kinins were strongly adsorbed by carbon, and indeed were irreversibly held by all but specially prepared carbons with low adsorptive power. While the kinins were adsorbed to such carbon, many salts and any remaining sugars could be removed by washing with water. Nearly all the ninhydrin-positive material and much coloured material were eluted by ethanol–ammonia. The kinins could then be recovered with the hot ethanol–ammonia–pyridine mixture.

The results of all three fractionations on the "Chromax" columns were similar. The separation achieved in solvent B was not as complete as that in solvent A, for paper-strip chromatograms suggested that peak III, IV of Figure 2 contained the kinins of peaks III and IV of Figure 1. It is possible that had the column been developed with more solvent B these two peaks would have been separated. No adequate explanations can be given for the apparent peaks 0 and II*a* (Fig. 2).

The results of all three fractionations strongly suggest that there are at least four independently active kinins in the apple extract.

There is as yet little evidence on possible relations between the kinins as might be revealed by interconversion. However, the results of acid hydrolysis suggest that peak III, IV kinins were partially converted to peak II kinin, but further work is needed to verify this point.

The area under each peak may be taken as a rough indication of the amount of each of the kinins present in the extract. The most plentiful would appear to be peak III, IV as shown in Figure 2. There are two reasons for this. Firstly, it contains both the low R_F kinins. The second reason arises from the method of application of extract to the columns. In those described by Figures 1 and 3 the extract was applied as a solution in solvent A, but the solubility of the peak IV kinin in this solvent is now known to be low. In the other column described by Figure 2 the entire extract was applied by drying onto filter paper.

By gradient elution from "Dowex 50" with hydrochloric acid, the kinins were separated from most of the ultraviolet-absorbing material. The subsequent development of the bulked active fractions on a paper column resolved the kinins, but spectrophotometric measurements failed to reveal a correlation between the peaks of activity and those of ultraviolet absorption. Therefore it seems that, if the kinins of apple fruitlets are ultraviolet-absorbing compounds, they must be in concentrations too low for spectrophotometric detection at this stage.

Beauchesne (1962) has summarized the properties of the kinin he has extracted from maize "milk". His assay with tobacco pith and a medium containing IAA is similar to ours and therefore it is possible to compare our results with his. He has shown that both his neutral fraction, which is the effluent from a mixed-bed resin, and his cationic fraction, which is eluted from the same mixed bed with ammonia, show kinin activity. He has purified the kinin from the cationic fraction. In contrast to our results he has found only one active material in this fraction. Like the apple kinins (Bottomley *et al.* 1963) it is insoluble in ether, is soluble in 90% ethanol, and passes through dialysis membranes. Unlike the apple kinin it is found in the effluent from a "Dowex 2"-OH^- column, but is similar in its strong adsorption to "Dowex 50"-H^+. Beauchesne has correlated this kinin with ultraviolet absorption and has concluded that it is a purine closely related to adenine.

Miller (1961) reported the detection of two kinins in an extract of "creamed maize". One of these was adsorbed to "Dowex 50"-H^+ and the other was found in the effluent. The adsorbed kinin was purified, and the most active preparation stimulated the growth of the assay tissue (soybean callus) at a concentration of 0·01 mg/l. Like our kinins it was displaced from "Dowex 50"-H^+ with ammonia and was eluted slowly with strong hydrochloric acid solutions. It was adsorbed by "Dowex 2"-acetate which does not adsorb the apple kinins. Miller was able to correlate the kinin activity with ultraviolet-absorbing spots on paper chromatograms and concluded that the observed absorption was due to the active substance.

The kinins of apple and maize are therefore similar in many major respects. The main difference is in the correlation of kinin activity with ultraviolet-absorbing material in extracts of maize and this correlation the present authors have been unable to achieve with apple extract.

Pollard, Shantz, and Steward (1961) have given some details of the methods used for their purification of the coconut-milk kinin. Whole coconut milk was passed through a column of activated carbon, which was eluted first with acetic acid and, after a water wash, with ammonium hydroxide. The combined acetic acid and

ammonium hydroxide eluates promoted the growth of carrot phloem explants at a concentration of 10 mg/l. There are insufficient data at present for comparisons to be made between the coconut and apple kinins.

V. Acknowledgments

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