MODIFICATION OF THE COURSE OF THE REACTION BETWEEN WHEAT FLOUR LIPOXYGENASE AND LINOLEIC ACID DUE TO ADSORPTION OF LIPOXYGENASE ON GLUTENIN

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SUMMARY: Lipoxygenase (E.C.1.13.1.13) is adsorbed on glutenin. The adsorption is reversible and complies with a Langmuir equation. The lipoxygenase-glutenin complex forms hydroxy-epoxy-acids from linoleic acid, which are rapidly hydrolysed to trihydroxy-acids. The adsorption is inhibited if the lipids of the glutenin are removed.

In a previous study we found that in wheat flour dough mixed under air or oxygen the reaction of lipoxygenase with linoleic acid may take two different courses (1): (a) A mixture of 13-and 9-hydroperoxyoctadecadienoic acids is formed; these are subsequently reduced to corresponding monohydroxy-acids; (b) A mixture of 9-hydroxy-10(trans),12,13(cis)-epoxy-octadecenoic acid and 9,10(cis)-epoxy-11(trans), 13 hydroxyoctadecenoic acid is formed, these are subsequently hydrolysed to 9,13-trihydroxy-10(trans)-octadecenoic acid and 9,10,13-trihydroxy-11(trans)-octadecenoic acid.

The present study revealed that in a lipoxygenase/gluten suspension, lipoxygenase is adsorbed on the glutenin fraction and that the adsorbed lipoxygenase is responsible for the second course.

MATERIALS AND METHODS

Flour:

For the isolation of glutenin, we used a commercial Dutch flour, milled from a blend of hard and soft bread wheats and not containing any bleaching or other improving agent; protein content (N x 5.7) 10.1 %. For the isolation of lipoxygenase we used a soft Dutch wheat (Manella). The grains were milled to 76 % flour extraction in a Brabender Quadrumat-Junior laboratory mill. The protein content of the flour was 15.2 %.

Isolation of gliadin and glutenin:

Flour (100 g) was extracted with 1 l of 0.014 M phosphate buffer (pH 6.8) in a Waring Blendor for 4 min. and the slurry centrifuged at 3000 x g. The supernatant was decanted and the residue extracted in a same

way five times with distilled water to remove the albumin, globulin, and gliadin fractions (2). It was shown by starch-gel electrophoresis that the first and second extractions contained mainly albumin and globulin proteins, whereas the fifth and sixth extractions contained mainly gliadin protein (more than 90 %)(3). The residue was freed of starch and water soluble material in a conventional way by hand-kneading in a continuous stream of distilled water. The resulting gluten ball was cut into small pieces and solubilized in 500 ml 0.05 N acetic acid. After centrifugation at 3000 x g to remove the acetic acid-insoluble material the solution was adjusted to pH 6.1 with 0.1 N sodium carbonate. The precipitated gluten was collected, washed with distilled water, and freeze-dried (5 g). This gluten preparation contained 85 % protein, consisting of 10 % ethanol-water (70 : 30 w/w)-soluble material (gliadin), 75 % glutenin and 10 % lipids (after acid hydrolysis).

Isolation of lipoxygenase fractions:

Portions of 6 g flour were homogenized with 60 ml distilled water in a Potter-Elvehjem homogenizer (4) and the suspension centrifuged for 20 min. at 300,000 x g in an M.S.E. superspeed 65 ultracentrifuge. The precipitate was discarded; the extract containing the lipoxygenase was freeze-dried. The freeze-dried material was fractionated on a Sephadex G-100 (Pharmacia, Uppsala, Sweden) column (7.0 x 40 cm). Elution was carried out with distilled water at 4 $^{\circ}$ (flow rate 20 ml/h). The lipoxygenase containing fraction represented by peak B in fig. 1 was freeze-dried (34 mg) and further fractionated on a CM-Sephadex C-50 column (2.0 x 30 cm) at 4 $^{\circ}$. Elution was successively carried out with 320 ml 0.016 M sodium phosphate buffer (pH 5.9), 180 ml 0.076 M sodium phosphate buffer (pH 7.5) and 120 ml 0.76 M sodium phosphate buffer (pH 7.5) containing 1 M NaCl. Two fractions, represented by peaks B_1 (8 mg) and B_4 (1 mg) in fig 2, showed lipoxygenase activity. The lipoxygenase activity per mg dry material of the fractions B, B_1 and B_4 was 12 x 10^3, 34 x 10^3 and 36 x 10^3 units, respectively.

Assay of lipoxygenase activity:

For measuring lipoxygenase activity the spectrophotometric method of Surrey (5) was used with slight modifications. Amounts of 0.01-0.05 ml of solution to be tested, were added to 2 ml substrate in a 1 cm cuvette; the absorbance was measured at 15 seconds' intervals at 234 nm with a Beckman spectrophotometer. As substrate a solution of linoleic acid (5 x 10^{-4} M) in 0.016 M phosphate buffer, pH 7.0, containing 500 mg Tween 20 per liter was used. One unit of enzyme activity is an absorbance increase of 0.001 per minute.

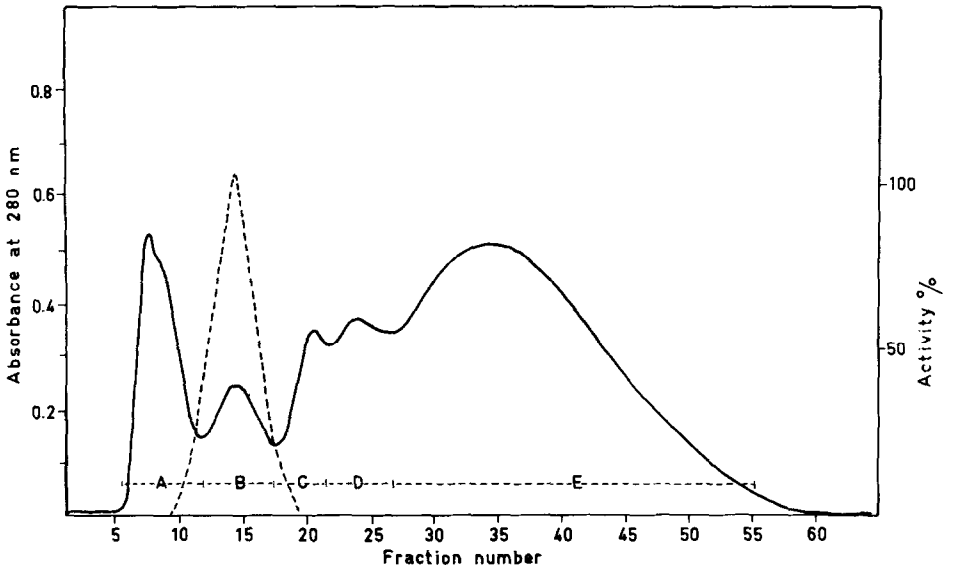

fig. 1. Gel-filtration of a water extract of wheat flour on Sephadex G-100. The dotted line indicates lipoxygenase activity.

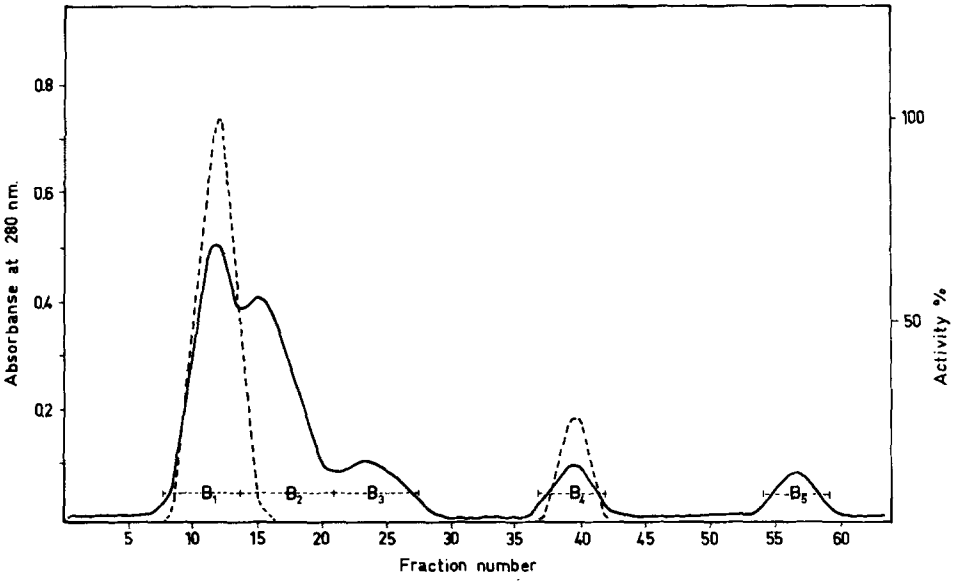

fig. 2. CM-Sephadex chromatography of active fraction B from Sephadex G-100 column. The dotted line indicates lipoxygenase activity.

EXPERIMENTS

Adsorption and desorption of lipoxygenase on gluten

The association between lipoxygenase and gluten was studied by adding 100 mg powdered freeze-dried gluten to 4 ml of the solutions containing quantities of lipoxygenase varying from 6.10^3 - 10^5 units. After 20 min. shaking at room temperature on a Vortex vibrating machine the suspension was centrifuged at 3000 x g. In the supernatant the lipoxygenase activity was measured. This experiment was carried out with the three lipoxygenase fractions, viz: B, B_1 and B_4. Fig. 3 shows the amounts of lipoxygenase

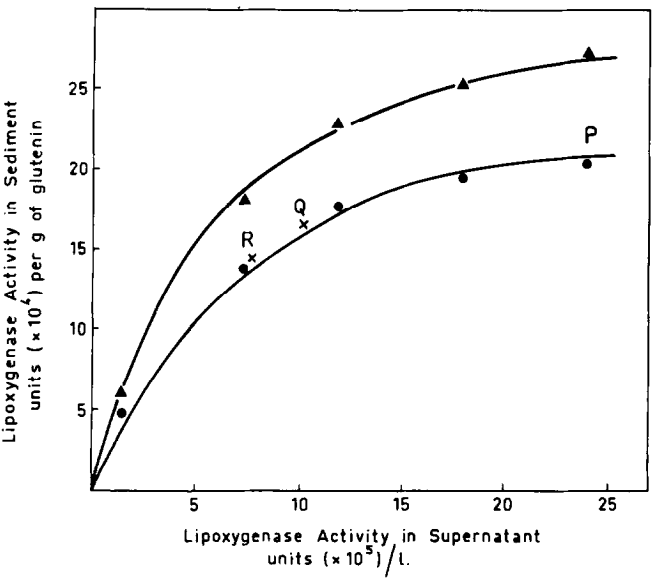

fig. 3. Adsorption isotherms of:
▲ Lipoxygenase from fraction B_1 or B_4.
● Lipoxygenase from fraction B.

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adsorbed to gluten plotted against the free lipoxygenase present in the liquid. Plotting the reciprocals of the adsorbed quantities against the reciprocals of the lipoxygenase activities in the liquid gives a straight line. Therefore, the adsorption isotherm can be described with a Langmuir equation:

$$x = S \frac{ac}{1 + ac}$$

in which x is the amount of lipoxygenase adsorbed per g of gluten, c is the equilibrium of lipoxygenase remaining in solution, S is the maximum adsorption, and a is a constant. From the plot of the reciprocals the following values for S and a were found:

	S (units per g of gluten)	a (l/unit)
B	26×10^4	1.5×10^{-8}
B_1	37×10^4	1.3×10^{-8}
B_2	37×10^4	1.3×10^{-8}

So, the maximum adsorption of fractions B_1 and B_4 is considerably greater than that of fraction B.

The adsorption is reversible. This is demonstrated by resuspending the sediment after centrifugation of the suspension containing the highest lipoxygenase concentration (Point P in fig. 3) in 4 ml water. The desorption proceeds slowly: after shaking for 2 hours equilibrium was attained (point Q). This suspension Q was centrifuged again and the sediment resuspended in 4 ml water (point R in fig. 3). The lipoxygenase can be completely removed from gluten by repeated washing with distilled water.

Determination of the oxidation products

To suspensions P, Q and R (fig. 3) 10 mg linoleic acid was added. After incubation for 1 hour the oxidation products were determined with the aid of thin layer chromatography (1). The results are given in Table I. This table shows that the ratio between adsorbed and non-adsorbed lipoxygenase

Table I

The ratio between adsorbed and non-adsorbed lipoxygenase and the ratio between the groups of oxidation products

Suspension	P	Q	R
$\frac{\text{Adsorbed lipoxygenase}}{\text{Non-adsorbed lipoxygenase}}$	2.1	4.1	6.0
$\frac{\text{Trihydroxy-acids}}{\text{Hydroperoxides + Monohydroxy-acids}}$	2.0	4.3	6.3

in the suspensions is the same as the ratio between trihydroxy-acids and hydroperoxides + monohydroxy-acids. This result corroborates the assumption that the non-adsorbed lipoxygenase is responsible for the production of hydroperoxides and mono-hydroxy-acids, whereas the adsorbed lipoxygenase forms trihydroxy-acids via hydroxy-epoxy-acids as intermediate products. Hydroxy-epoxy-acids, however, were not found as in a suspension they are immediately hydrolysed to trihydroxy-acids (1).

Table II

Effect of glutenin extraction on the adsorption

Extraction solvent	Adsorption *
Not extracted	26×10^4
Aceton	23×10^4
Chloroform	20×10^4
Petroleum ether	18×10^4
70 % ethanol	14×10^4
Chloroform--methanol 2:1	5×10^4

* Maximum adsorbed lipoxygenase in units per g of glutenin

Adsorption of lipoxygenase on defatted, heated or reduced gluten:

For further study of the adsorption we extracted gluten by percolation with various organic solvents, determined the adsorption isotherms and calculated therefrom the maximum adsorption in suspensions of 100 mg defatted gluten in 4 ml water. For these experiments the lipoxygenase-containing fraction B was used. The results are given in Table II. The organic solvents cause a decrease of the adsorption maximum. The more polar the solvent, the greater the effect.

An even greater effect was found after heating the gluten. 100 mg gluten suspended in 4 ml water was heated at 100 $^{\circ}$ for 5 min. After cooling lipoxygenase of fraction B (10^5 units) was added. No lipoxygenase was adsorbed on the heated gluten.

The adsorption capacity disappeared also after chemical modification of the gluten. To a suspension of 100 mg gluten in 4 ml water 3 mg $NaBH_4$ was added and the suspension was shaken in nitrogen. As a result of reductive decomposition no lipoxygenase was adsorbed. If the suspension was subsequently shaken in air for 30 min, as a result of the re-oxidation the lipoxygenase was adsorbed again. In contrast, if, in addition to $NaBH_4$ 5 mg N-ethylmaleimide was added to block the thiol-groups no adsorption could be detected after re-shaking in air.

Adsorption of lipoxygenase on gliadin:

To a solution of 100 mg gliadin (obtained by fifth and sixth water extractions of flour) in 4 ml water 10^5 units lipoxygenase (fraction B) was added. The gliadin remained completely in suspension even after centrifugation; it was therefore impossible to determine the adsorption of lipoxy-

genase. The oxidation products are determined after addition of 10 mg linoleic acid and shaking in air for 1 hour. Only hydroperoxides and monohydroxy-acids are formed. From these facts it may be concluded that no association between lipoxygenase and gliadin has taken place.

DISCUSSION

The experiments have shown that lipoxygenase is adsorbed on gluten. The adsorption is reversible and complies with a Langmuir equation. Besides, it was found that the lipoxygenase-gluten complex forms hydroxy-epoxy-acids, which are rapidly hydrolysed to trihydroxy-acids, as reported earlier (1).

As no trihydroxy-acids are formed from linoleic acid in a mixture of lipoxygenase and gliadin, it is reasonable to assume that the glutenin fraction of the gluten adsorbs lipoxygenase.

Recent investigations have revealed that the glutenin fraction adsorbs also proteases (6) and β-amylases (7). It is possible that in our experiments these enzymes competed with lipoxygenase for a place on the glutenin.

The adsorption isotherms (Fig. 1) show that the purer the lipoxygenase the more of it is adsorbed. Apparently, other water-soluble materials are able to inhibit the adsorption on glutenin. Besides, it is probable that further purification of the glutenin may enhance its capacity to adsorb lipoxygenase.

The experiments have shown that the adsorption of lipoxygenase on glutenin is partly or completely barred if the glutenin fraction has been heated or the lipids have been removed. Reconstituting the glutenin by adding the removed lipids does however not restore its ability to adsorb lipoxygenase. The adsorption of lipoxygenase is lost at reductive breakdown of the glutenin; in this case adsorption may be restored by re-oxidation.

In a previous publication (1) we pointed out that in a dough relatively more hydroxy-epoxy-acids and trihydroxy-acids are formed than in a flour/water suspension. This may be accounted for by assuming that in a dough more lipoxygenase is adsorbed on glutenin than in a suspension. This fact has probably to be ascribed to two factors: to the effect of kneading, and to the scarcity of free water in a dough (8,9). The latter circumstance offers a reasonable explanation of the slower hydrolysis of the hydroxy-epoxy-acids in a dough than in a suspension.

It will be interesting to know whether the adsorption of lipoxygenase on glutenin also takes place during ripening and/or during germination of wheat kernels, and whether the adsorption affects the metabolic function of the lipoxygenase in the kernels.

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