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Isolation and physicochemical properties of an adenosine-rich gluten fraction

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Gluten proteins were isolated from the 0.01 mol acetic acid extract of bread. It was observed that precipitation of gluten provoked by 200 mM NaCl could partly be inhibited by adenosine. Based on this finding a method for isolation of the gluten fraction resisting saline precipitation in the presence of adenosine was elaborated. This fraction termed by us gluten-A-S, was found to have a lower glutamine + glutaminic acid and a higher proline and phenylalanine content than gluten. By sodium dodecylsulphate polyacrylamide gel electrophoresis gluten-A-S was shown to contain components of 58 000 and 34 000 dalton molecular weight after mercaptoethanol treatment while without the latter it contained a component of 74 000 dalton. The absorption maximum of the compound is at 260 nm; $E^{280nm}/E^{260nm} = 0.5$. In accordance with previous findings, gluten-A-S was found to contain 50–100 nmol adenosine per mg protein in a strong binding.

It seems that in addition to the small amount of tightly bound adenosine, gluten-A-S contains a larger quantity of adenosine loosely bound to the protein.

The physiological effects of dissociable adenosine bound to gluten and its possible role in the pathomechanism of gluten sensitive enteropathy is discussed in detail.

In previous studies it was shown that certain fractions of gluten proteins (gliadine, glutenine) fail to precipitate under the effect of 200 mM NaCl if various purine derivatives (caffeine, adenosine, adenosine triphosphate, etc.) are added [11], in other words the gluten proteins become partly soluble probably as a result of interaction between them and the purine derivative.

Adenosine isolated from gluten extracts has been incriminated for the inhibitory effect of gluten proteins on intestinal peristalsis [8]. Since gluten preparations were found to contain purine or pyrimidine derivatives [10] it seemed logical that protein-bound purine or pyrimidine derivatives liberated in the intestine might slow down bowel movements by inhibiting acetylcholine production, i.e. they might play some role in the pathomechanism of coeliac disease.

For this reason we examined the nature of gluten protein — adenosine bonds by measuring some physicochemical properties (molecular weight, absorption spectrum, amino-acid composition, etc.) of the gluten fraction resisting precipitation by 200 mmol/l NaCl in the presence of adenosine. In this paper the results of that study are described.

Materials and Methods

Gluten was used in the experiments as in a previous study [11] the highest quantities of protein were found in the supernatant after precipitation by 200 mmol/l NaCl in the presence of adenosine, if non-fractionated gluten was used.

Preparation of gluten from bread. Slices of 200 g wheat bread were extracted by 1000 ml of a solution containing 10 mmol/l acetic acid and 0.8 mmol/l sodium azide at room temperature under continuous stirring for four hours. The mixture was then kept at 4 °C for 14–16 h, thereafter centrifuged at 2000 *g* at 4 °C, for 60 min. The precipitate was discarded, the volume of the supernatant was measured, and under continuous stirring solid sodium chloride was added to a final concentration of 200 mmol/l. The mixture was kept at 0 °C for 4–5 h, thereafter centrifuged at 2000 *g* for 60 min. The supernatant containing mainly starch and saline soluble proteins was discarded, the precipitate was redissolved in about 100 ml of the extraction solution, then solid NaCl was added up to a final concentration of 200 mmol/l. After standing for some hours, centrifugation, discarding the supernatant, redissolution of the precipitate in acetic acid plus sodium azide, addition of salt, and centrifugation were repeated. The last precipitate was dissolved in the extraction fluid in a quantity to obtain a protein concentration of 3–5 mg/ml. In order to remove the undissolved proteins the mixture was centrifuged at 40 000 *g* for 40 min (Beckman L3-50 preparation centrifugue, Ti 60 rotor). The precipitate was discarded and the clear supernatant containing the gluten protein was kept at −20 °C until used.

Adenosine treatment of gluten protein. To 36 ml solution containing 0.6–0.7 mg protein/ml, 310 mg adenosine was added. To promote solution of the latter, the mixture was continuously stirred at 37 °C, the final concentration of adenosine was thus 30 mmol/l. The solution was kept at room temperature for 30 min, 36 ml 400 mmol/l NaCl was added, the mixture was kept in ice-water for 2–4 h and then centrifuged at 40 000 *g* at 0 °C for 40 min. The precipitate was redissolved in a solution containing 10 mmol/l acetic acid and 0.8 mmol/l sodium azide and dialysed against the same fluid in order to remove the excess adenosine. The precipitated protein was termed gluten-A-P, the supernatant protein gluten-A-S. To remove the remaining adenosine, the mixture was dialysed 8 times against 2 l of ion-free water which was changed first after 4 h, then after each twelve hours.

Demonstration of adenosine in gluten-A-S was carried out by paper chromatography using isopropanol-ammonia-water, 7 : 2 : 1. After chromatography the paper was dried, the spot of adenosine clearly separated from protein was localised under UV-light, cut out and eluted in 0.1 N HCl. The concentration of adenosine was determined by measuring optical density at 259 nm, and the adenosine content in nmol of 1 mg gluten-A-S was calculated.

Amino acid analysis. The amino acid composition of all preparations was determined by help of a Lys (Chinoin) amino acid analyser as described previously [10].

Sodium dodecylsulphate polyacrylamide gel electrophoresis at pH 7. Samples containing 50–100 mg protein were mixed in a ratio of 1 : 1 or 1 : 2 with incubation solution containing 0.01 M sodium phosphate pH 7, 5% sodium dodecylsulphate, 1% mercaptoethanol ("+MCE") or without this latter compound ("−MCE"), 0.005% bromophenol blue and 40% glycerol; these were incubated at 100 °C for 5 minutes, quantitatively transferred under electrode buffer onto the top of the gel columns. Electrophoresis, staining and removal of excess stain were carried out according to Weber and Osborn [13]. The reference proteins used as standards were, bovine serum albumin (molecular weight: 68 000 dalton), IgG ("+MCE"): 50 000 resp. 23 000, "−MCE": 160 000 dalton), and ovalbumin (molecular weight: 42 000 dalton) as described in detail elsewhere [10].

Determination of absorption spectra and protein content. The absorption spectrum of proteins was examined in an Opton PM 2 DL spectrophotometer. The protein content was measured by the method of Lowry et al [6]. The calibration curve was constructed by the use of known quantities of bovine serum albumin. All determinations were carried out in 4–5 parallel samples. The results were nearly identical, in the Table and Figures one typical experiment each is shown.

All reagents were of analytical purity produced by Reanal (Budapest), with the exception of adenosine which was a product of Serva (Heidelberg).

Results

In the first series of experiments the effect of various adenosine μmol/protein mg ratios on the precipita-

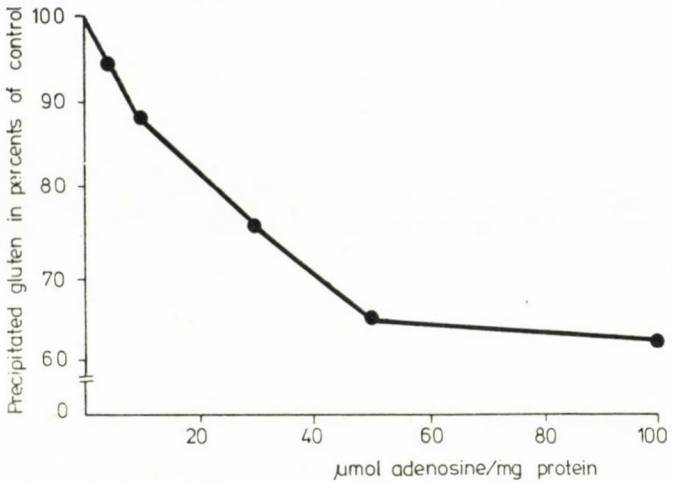

FIG. 1. Precipitation of gluten by 200 mM NaCl in the presence of various μmol adenosine/mg protein ratios. See text for details

tion of gluten in 200 mmol/l NaCl was investigated. Samples containing gluten, sodium chloride and various amounts of adenosine were centrifuged at 2000 *g* for 60 minutes and the protein content of the precipitate was determined [6]. As can be seen from Fig. 1, precipitation of gluten by 200 mmol/l NaCl decreased with the increase of the adenosine/protein ratio from 0 to 50 μmol/mg. Over 50 μmol/mg there was no further change, so that in subsequent experiments a ratio of 45–50 μmol/mg protein was applied.

Table I shows the results of amino acid analysis. The composition of gluten-A-P is similar to that of non-fractionated gluten and this explains the fact that like gluten-gluten-A-P is soluble only in acetic acid. Gluten-A-S greatly differs from both gluten-A-P and gluten in this respect. In gluten-A-S there is less glutaminic acid + glutamine, while its proline content exceeds 1.4 times and its phenylalanine content nearly twice the corresponding values of gluten. The gluten–A–S preparation too has

TABLE I

Amino acid composition of gluten, gluten-A-P and gluten-A-S

Amino acid	Gluten	Gluten-A-P	Gluten-A-S
	mol per cent		
Lys	2.30	2.6	0.73
His	1.10	1.1	1.36
Arg	1.60	1.7	0.94
Asp+Asn	2.00	1.5	4.38
Thr	1.80	1.7	2.76
Ser	4.80	4.8	6.81
Glu+Gln	42.10	40.0	34.3
Pro	17.90	19.7	24.3
Gly	2.90	3.8	2.89
Ala	2.40	2.4	1.1
Val	3.00	2.3	1.3
Met	0.90	0.8	0.87
Ile	3.60	3.8	2.78
Leu	5.90	5.4	4.42
Tyr	2.70	1.8	1.1
Phe	5.00	5.7	9.7

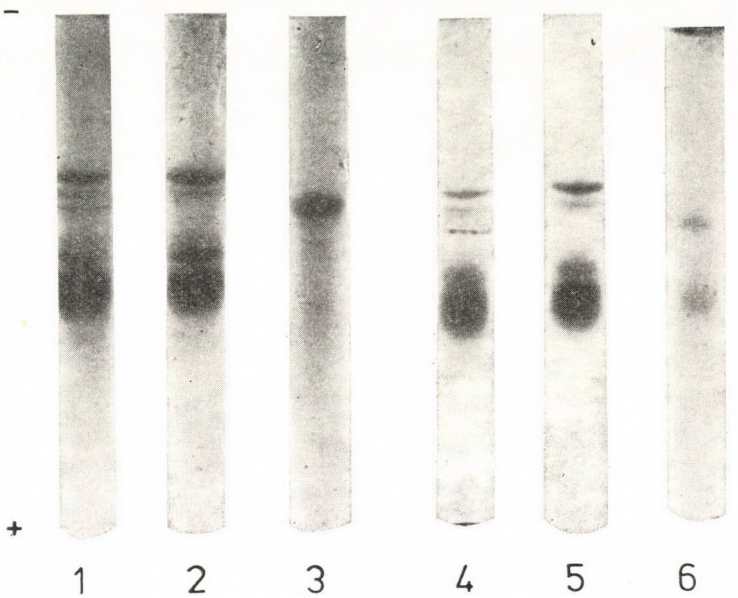

FIG. 2. Stained preparations of gluten (1,4), gluten-A-P (2,5) and gluten—A-S (3,6) after sodium dodecylsulphate polyacrylamide gel electrophoresis. Pictures 4, 5 and 6 represent preparations pretreated with heat (100 °C) and mercaptoethanol for 5 minutes. With preparations 1—3 no mercaptoethanol was applied

a different solubility: it is soluble in water and saline.

The preparations were tested for homogeneity by sodium dodecylsulphate polyacrylamide gel electrophoresis. The first three sections of Fig. 2 show the composition of preparations heat treated in an incubation fluid not containing mercaptoethanol ("—MCE"); the second three sections

TABLE II

Molecular weight of components obtained by sodium dodecylsulphate polyacrylamide gel electrophoresis of gluten, gluten-A-P and gluten-A-S

Preparation	Incubation fluid	Component						
		1	2	3	4	5	6	7
		Molecular weight, 10^3 dalton						
Gluten	−MCE	97	83	74	56	49	40	34.5
	+MCE	74	66	60	49	43	38	32.5
Glu-A-P	−MCE	97	82	—	56	48	39	34.5
	+MCE	73	65	—	47	43	36	32.5
Glu-A-S	−MCE	—	—	74	—	—	—	—
	+MCE	—	—	58	—	—	34*	31*

* traces

show the corresponding pictures obtained in "+MCE" experiments; here the eventual disulphide bonds have been subjected to reduction. The results of both types of pretreatment are summarized in Table II. This shows the following facts. On electrophoresis gluten separated into seven components different in molecular weight. Pretreatment with mercaptoethanol did not increase their number, but changes occurred in their molecular weight. This points to changes in molecular shape induced by disulphide bond reduction within the polypeptide chain, resulting in a decrease of molecular weight, as it has already been assumed [1, 12]. The third fraction counted from the top of the gel column of non-fractionated gluten, which originally had a molecular weight of about 74,000 dalton and 5800 respectively 38,000 dalton after mercaptoethanol reduction, appeared in the supernatant (gluten-A-S) and was thus missing from gluten-A-P.

Polyacrylamide gel electrophoresis without SDS treatment by the method of Davis [3] described in detail earlier [2] revealed that the gluten-A-S protein (Fig. 3, 3) mainly consists of gluten fraction I (Fig. 3, 1) sited next the cathode. Correspondingly, gluten-A-P (Fig. 3, 2) contains comparatively small amounts of fraction I while fractions II and III dominate in it.

The absorption spectrum of gluten-A-S and gluten-A-P was examined and compared with that of non-fractionated gluten (Fig. 4). The maxi-

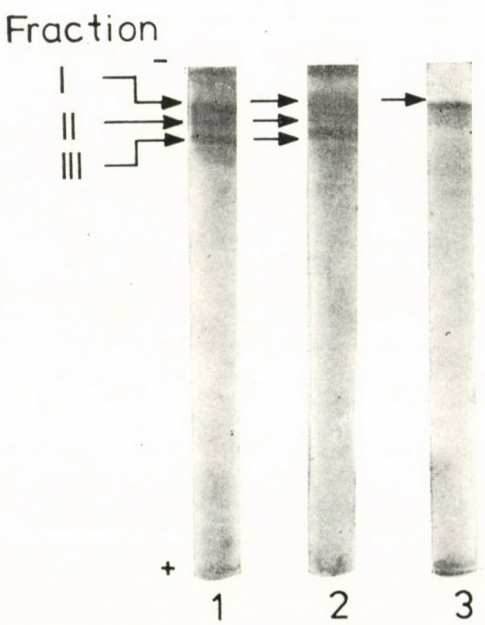

FIG. 3. Testing for homogeneity of gluten (1), gluten-A-P (2) and gluten-A-S (3) by polyacrylamide gel electrophoresis without sodium dodecylsulphate, according to the method of Davis (3)

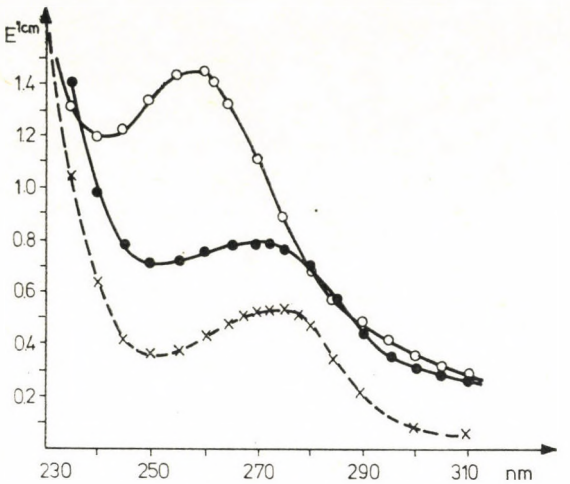

FIG. 4. Absorption spectra of gluten (crosses), gluten-A-P (dots) and gluten-A-S (circles)

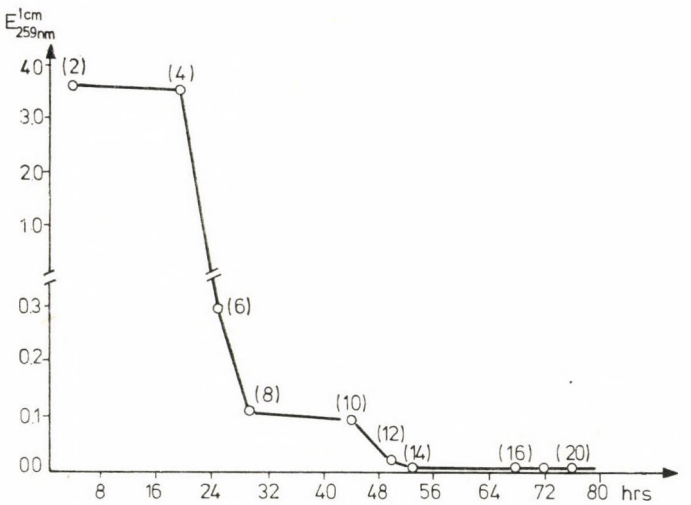

FIG. 5. Extinction values measured at 259 nm in the dialysing water of gluten-A-S, plotted against time and volume of dialysing fluid. The numbers in brackets next the points representing the individual extinction values indicate the cumulative volume of dialysing fluid

mum of gluten and gluten-A-P lies at 275 nm while with gluten-A-S the highest extinction was found at 260 nm. This suggests that the adenosine binding component is in the supernatant. As described in the methodological section, 1 mg gluten-A-S contains 50–100 nmol adenosine.

When dialysis was performed in order to remove adenosine, about 70–72 ml of the supernatant was dialysed against 2 l cold ion-free water

first for four hours, then seven more times for 12 hours each. The optical density of each 2 liter dialysis fraction was measured at 259 nm in order to determine its adenosine content. Fig. 5 shows the results; here the extinction values are plotted against time, the numbers in bracket represent the cumulative volume of the dialysing water. It can be seen that the adenosine concentration did not decrease gradually between the 4th (8) and 5th (10) fractions and adenosine was not practically removed from the gluten-A-S, while between the 5th (10) and 6th (12) fractions the adenosine concentration decreased nearly to zero. From this it was concluded that gluten-A-S contains adenosine combining sites different in strength.

Discussion

It has been shown [10] that gluten protein preparations contain purine or pyrimidine derivatives (nucleic acids, nucleotides, nucleosides or bases). Robinson et al [8] isolated adenosine from gluten extract by chromatography and electrophoresis and showed that it was the factor responsible for the inhibitory effect of gluten proteins on intestinal peristalsis. It may thus be anticipated that purine or pyrimidine derivatives bound to gluten proteins and liberated in the bowel are capable of inhibiting acetylcholine production and in this way intestinal motility may play some intermediary role in the pathomechanism of coeliac disease.

Salt precipitability of gluten proteins decreases when adenosine is added to the medium (Fig. 1). This led us to the idea that a protein component containing adenosine and soluble in saline could be isolated. Such a fraction may be useful in immunological and inhibition experiments where solubility at various pH values is an important requisite.

We succeeded in isolating a gluten fraction soluble in saline at various pH values by salt fractionation of gluten in the presence of adenosine. This fraction, gluten-A-S, differs from gluten and gluten-A-P by its amino acid pattern: it contains less glutamine and glutaminic acid and more proline and phenylalanine (Table I). Also, the sum of apolar amino acid (Pro, Gly, Ala, Val, Met, Ile, Leu, Phe) concentrations is higher in gluten-A-S (47.7%) than in gluten (41.6%) or gluten-A-P (43.9%). In spite of the higher participation of apolar amino acids, gluten-A-S has a better solubility in water or saline.

Sodium dodecylsulphate polyacrylamide gel electrophoresis revealed the presence of a component with a molecular weight of 74,000 dalton and, after pretreatment with mercaptoethanol, two fractions weighing 58,000 and 34,000 dalton, respectively, were obtained.

The absorption maximum of gluten and gluten-A-P is at 275 nm, that of gluten-A-S at 260 nm. The latter may be due to the adenosine bound to gluten-A-S, or to a high participation of phenylalanine in the protein structure. The second possibility can be

excluded since a high phenylalanine content leads to four maxima, at 253, 259, 265 and 269 nm [4]. Since the gluten-A-S preparation has a single maximum at 260 nm and the presence of adenosine in a quantity of 50 nmol/mg protein can be demonstrated, it may be regarded as proven that gluten-A-S (with a ratio $E^{280nm}/E^{260nm} = 0.5$) does in fact contain adenosine.

In some preliminary experiments the eventual inhibitory effect of gluten-A-S was tested; it exerted no or hardly any effect on the acetylcholinesterase of the fragmented sarcoplasmic reticulum isolated from the muscle tissue of the fish Amiurus nebulosus (this preparation has a very high enzyme activity: 1–2 μmol acetylcholine per mg protein/minute) [9], nor on the adenosine-triphosphatase activity of actomyosin isolated from rabbit muscle by the method of Portzehl et al [7]. It is also probable that the adenosine content of the gluten-A-S fraction, which is released from its binding in the intestinal lumen, is not sufficient to interfere with acetylcholine production and, consequently, with intestinal peristalsis. We suppose that much more adenosine would be needed for such an effect. However, in addition to the non-dialysable adenosine, gluten-A-S probably contains a fairly large amount of adenosine which is easily liberated from the protein molecule, as can be judged from Figure 5. This rapidly released adenosine may exert an inhibitory effect on acetylcholine production and intestinal motility.

Our finding concerning a large quantity of adenosine loosely bound to gluten-A-S and its possible effect on peristalsis needs experimental confirmation by studies on the adenosine binding sites of gluten, their number, quality and binding constants.

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