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Effect of a calcium-binding gluten fraction on the superprecipitation of actomyosin

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The Ca^{2+}-binding gluten fraction isolated by the authors [34] has been shown to prolong the clearing phase and to shorten the physiological contraction phase of ATP activated natural actomyosin suspension in the presence of physiological potassium chloride in a dose dependent manner. The mean ATPase activity of myosin and actomyosin was calculated for each phase from the actual ATP concentrations, measured at 30 sec intervals, and from the phase lengths. The preparation was found markedly to inhibit the ATPase activity during the clearing and physiological contraction phase. Since both myosin and actomyosin ATPase is Ca^{2+}-dependent, it is assumed that the inhibitory effect of the gluten fraction on ATPase activity may be mediated by its free Ca^{2+}-binding capacity, resulting in modified phase lengths of actomyosin superprecipitation.

On the basis of these experimental results a hypothesis is put forward of the part of the Ca^{2+}-binding gluten fraction played in the pathomechanism of coeliac disease.

Since Dicke's observation made in 1950 [11] the causal role of wheat and rye gluten in the aetiology of coeliac disease has been accepted, although the exact pathomechanism has not been clarified. There are strong arguments for a biochemical [5, 6, 7, 8, 21, 27, 28, 31, 32, 33, 34] and an immunological mechanism [4, 10, 13, 14, 15, 17, 18, 19, 20, 23, 30, 31] alike. In either concept the initial step in gluten toxicity is binding of this protein to the mucosa. This binding either acts directly on the enterocytes perhaps by gluten endocytosis into the lysosome [27, 28], or provokes an immunological reaction to gluten or its fragments [10, 23, 30]. Such a reaction might be ascribed to abnormal permeability of the intestinal mucosa [1, 2, 3] and to the unique amino acid composition of gluten, rendering this protein resistant to proteases [30]. It is known that certain peptide bindings, e. g. glycyl-glycine, prolyl-peptides (we have found 17—20% proline in gluten on a molecular basis) are resistant, while other bindings like lysyl-glutaminic acid, arginyl-glutaminic acid, etc., are partly resistant to pancreatic and intestinal proteases [24].

We have recently isolated some gluten fragments that possess the capacity of altering the concentration of certain compounds acting under physiological conditions [32, 33, 34]. These gluten components may play a

secondary or even a primary role in the pathomechanism of coeliac disease.

In a previous paper [34] we reported on a gluten fraction capable of binding calcium; we termed this compound as gluten ES. This preparation may influence the level of free Ca^{2+}; thereby, it may be anticipated that it can inhibit biological processes needing the regulatory effect of free Ca^{2+}. In fact, we have demonstrated that gluten ES inhibited the $Ca^{2+} + Mg^{2+}$-dependent ATP-ase activity and Ca^{2+} uptake of the fragmented sarcoplasmatic reticulum isolated from rabbit striated muscle. More recently, we have studied the quantitative effect of gluten ES on another Ca^{2+}-mediated system regulating the length of individual phases and the $Ca^{2+} + Mg^{2+}$-dependent ATP-ase activity during these phases of actomyosin superprecipitation. In the experiments we used the model of phasic superprecitation of natural actomyosin induced by ATP in the presence of 140 mm l/l potassium chloride [9, 16, 36].

Material and Methods

Actomyosin was isolated from rabbit striated muscle according to Ebashi's method [12]. Gluten ES was prepared as described in a previous paper [34].

Follow-up of the superprecipitation was carried out as follows.

Composition of the reaction mixture of 3 ml: 140 mmol/l KCl, 20 mmol/l TRIS-HCl, pH = 7.0, 0.77 mmol/l $MgCl_2$, 0.077 mmol/l $CaCl_2$, 1 g/l actomyosin, 1 mmol/l ATP.

The reaction was initiated by addition of ATP. Changes in turbidity were continuously registered at 660 nm wave-length by a compensograph OH-814/1 (Radelkisz) connected with a spectrophotometer Spektromom 195 (MOM, Budapest); the reaction mixture was continuously mixed by a magnetic mixer in a cuvette of 1 cm depth, its temperature was kept at 25°C.

The time course of ATP concentrations during superprecipitation was studied as follows.

20 μl samples of the reaction mixture were removed at 30 sec intervals and added to 180 μl 100 g/l trichloroacetic acid in order to stop the reaction. The samples were neutralized by 0.2 mol/l TRIS, then buffered in 0.1 mol/l TRIS-acetate-EDTA buffer (pH = 7.75), luciferin luciferase (LKB) was added and the bioluminescent light intensity (I) dependent of the concentration of ATP was measured in a Packard TRI-CARB 3320 liquid scintillation spectrometer. The system was calibrated for the concentration range 10^{-5}—10^{-8} mol/l ATP [26]. The calibration line was fitted to a function of $lg(ATP) = a_0 + a_1(lg\ I) + a_2(lg\ I)$ type. Protein was measured by the method of Lowry et al [22], bovine serum albumin (SERVA) was used as the standard.

Results

Gluten ES prolonged the clearing phase of actomyosin superprecipitation in a dose-dependent manner (Figure 1). For better understanding tangential lines were drawn to each section of the registrate and their section points were projected to the abscissa representing the time axis. Thereby we obtained the time span of each period. The approximately horizontal phases showing no appreciable change in the turbidity of the

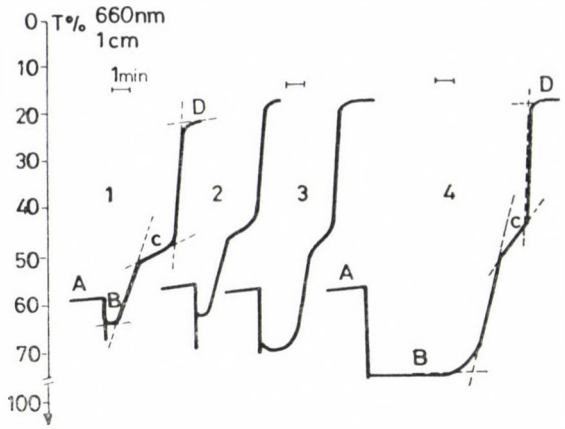

FIG. 1. Effect of bovine serum albumin (BSA) and various quantities of gluten ES on the superprecipitation of actomyosin. Changes in light transmittance of actomyosin in controls (1), after addition of 100 μg BSA (2), 30 μg gluten ES (3) resp. 80 μg gluten ES (4). A: Associated state of actomyosin; B: clearing phase; C: phase of physiological contraction; D: supercontraction phase

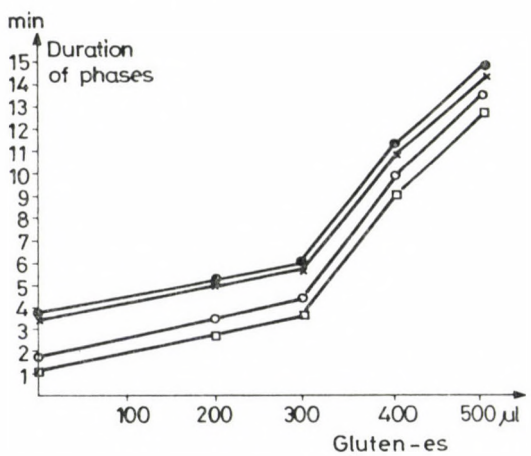

FIG. 2. Effect of various quantities of gluten ES on the length of phases of actomyosin superprecipitation. □——□: clearing phase; ○—○: onset of physiological contraction; x — x: end of physiological contraction phase; . —.: onset of supercontraction

actomyosin suspension were labelled A, B, C and D; A: actomyosin in the state of association; B: clearing phase (actomyosin is dissociated); C: phase of physiological contraction; D: phase of supercontraction.

Further, we studied the effect of increasing concentrations of gluten ES on the phase lengths within superprecipitation. Figure 2 demonstrates that the higher gluten ES content in the reaction mixture prolongs the clearing phase (B). On the other hand, the period extending between the beginning and the end of the physiological contraction shortens

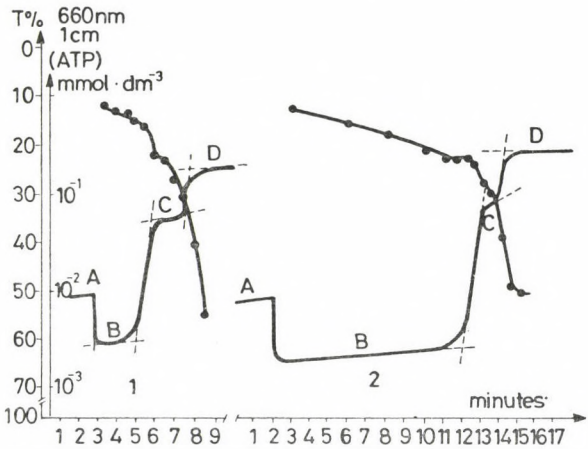

FIG. 3. Changes in ATP concentration and light transmittance in the course of actomyosin surperprecipitation. Controls (1); 80 μg gluten ES (2). —: change in transmittance; -•-•-•-•- change in ATP concentration

with increasing gluten ES concentrations (phase C).

To have a better insight into the effect of gluten ES on superprecipitation, we also studied the actual ATP concentrations during the individual phases. It is known from the literature [35] that actomyosin is in the state of association before starting the reaction by addition of ATP (Figure 3, A). In the moment of initiation of the reaction by ATP 1 mmol/l, this compound splits actomyosin to actin and myosin (clearing phase, in Figure 3: B). The changes in the actual ATP level observed from the beginning of the clearing phase are illustrated in Figure 3. In the control experiment this value is 0.576 mmol/l at the end of the clearing phase (Figure 3, 1), i.e.

TABLE I

Mean ATP-ase activity during each phase of superprecipitation versus gluten ES quantity

Water added to the reaction mixture, μl	Gluten ES	ATP-ase activity, nmol ATP · protein mg^{-1} · min^{-1}			
		Phase B	Transition from phase B to phase C	Phase C	Transition from phase C to phase D
		(phase length, min /activity (actual ATP concentration at the end of the phase, μmol ATP/l)			
500	—	(2.30)	(0.70)	(1.50)	(0.35)
		184	411	133	114
		(576)	(288)	(88)	(48)
200	300	(2.80)	(0.70)	(1.25)	(0.47)
		174	360	115	162
		(512)	(260)	(116)	(40)
—	500	(9.9)	(0.90)	(0.75)	(0.45)
		76	100	80	124
		(242)	(152)	(92)	(36)

the decrease is 0.424 mmol/l. In the presence of 500 μl (80 μg) gluten ES, 0.758 mmol/l ATP is consumed during a clearing phase 4.3 times longer than in the control experiment. From the phase length and the serial ATP measurements we calculated the mean ATP-ase activity of myosin and actomyosin during the individual phase. In the presence of gluten ES ATP-ase activity had markedly changed during the clearing phase and in the transitional period between the end of the clearing phase and the beginning of the physiological contraction phase (Table I). Since actomyosin dissociated at the starting point of the clearing phase and myosin ATP-ase is Ca^{2+}-dependent and actomyosin ATP-ase is $Ca^{2+} + Mg^{2+}$-dependent, it seems likely that gluten ES inhibits ATP-ase activity by reducing the free Ca^{2+} level.

Discussion

It is generally accepted that contraction of glycerol treated muscle fibres, myofibrils and actomyosin need not only Mg^{2+} and ATP but also calcium [25, 37]. In other words the state of contraction and relaxation of the contractile system is regulated by the free calcium ion concentration of the sarcoplasm. In relaxed muscle this has a value of 10^{-7} to 10^{-8} mol/l. An increase of the free Ca^{2+} concentration to $10^{-5} - 10^{-6}$ mol/l triggers contraction [25, 29, 37]. The gluten fragment isolated by us may influence muscular function by virtue of its calcium-binding capacity, the function of intestinal smooth muscles included, resulting in hypotonia of the intestine observed in coeliac disease.

To scrutinize this hypothesis we studied the effect of gluten ES in detail on a system representing an elementary process of muscle contraction. It has been long known that the so-called natural actomyosin suspension prepared from striated muscle tissue clears up if a high concentration of ATP (1—5 mmol/l) is added; the protein precipitates again if the ATP level decreases to 0.1 mmol/l as a result of enzymic cleavage of ATP. This phenomenon was termed as superprecipitation [35]. In the presence of physiological potassium chloride (140 mmol/l) the superprecipitation of actomyosin shows several phases [9, 16, 36], the clearing phase is followed by the physiological contraction phase, this in turn by a second phase of precipitation, the supercontraction phase. This phasic process was used in our experiments. In addition to the protein of the thick filament, myosin, our actomyosin preparation also contained actin, tropomyosin and the troponin complex, proteins of the thin filament. Tropomyosin binds the troponin complex to the thin filament. The troponin complex consists of three components: troponin-T, a compound responsible for binding to tropomyosin; troponin-C, a compound showing calcium ion binding property; and troponin-I, which inhibits the interaction between action and myosin. The key

step of contraction in this system is hydrolysis of ATP, a process carried out by Ca^{2+}-activated myosin ATP-ase and/or $Ca^{2+} + Mg^{2+}$-activated actomyosin ATP-ase.

In our experiments gluten ES prolonged the clearing phase, thus delaying the onset of physiological contraction, and shortened the latter phase (Figures 1 and 2). In our opinion, this prolongation of the clearing phase induced by the presence of gluten ES, cannot be fully explained by mere calcium ion detraction causing inhibition of myosin and actomyosin ATP-ase activity. It can be seen in Table I that in the presence of gluten ES markedly larger quantities of ATP are broken down by the end of the clearing phase than in the control; it is true that this more intensive ATP degradation occurs during a markedly prolonged phase. It may be anticipated that the decreased free calcium ion concentration induced by gluten ES not only inhibits ATP-ase activity but also leads to a delay in saturation of troponin-C with Ca^{2+}, an important prerequisite of contraction. We think that gluten ES probably shortens the phase of physiological contraction because, as it can be seen in Table I, the actual ATP concentration at the beginning of the physiological contraction is lower (260 resp. 152 μmol/l) than in the control experiment (288 μmol/l).

We have thus shown that the fraction of gluten isolated by us is capable of influencing muscle function. Although the experiments were carried out on actomyosin preparations taken from striated muscle tissue, it appears plausible that the same mechanism may act in intestinal wall hypotonia, a phenomenon characteristic of coeliac disease. In addition to the decreased muscle tone, reduced peristalsis may also result, as a consequence of the action of purine and pyrimidine derivates bound to another fraction of gluten [32, 33] and inhibiting cholinergic transmission and intestinal peristalsis if released.

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