LECTIN FROM EMBRYONIC CHICK MUSCLE THAT INTERACTS WITH GLYCOSAMINOGLYCANS

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1. Introduction

Extracts of embryonic chick muscle contain two hemagglutinins, referred to as lectins, that are inhibited by specific saccharides and that change strikingly in specific activity with muscle development [1,2]. The first to be characterized, a lactose-sensitive agglutinin here designated lectin-1, is a dimeric protein with subunit mol. wt $\sim 15 \times 10^3$ [3,4] and is also found in other chick tissues [5,6]. Similar proteins have been observed in extracts of organs of other species [7,8]. The second lectin from embryonic chick muscle, here designated lectin-2, does not agglutinate the trypsinized, glutaraldehyde-fixed rabbit erythrocytes used to assay lectin-1; but will agglutinate some samples of these test cells that had undergone transformation upon storage in the refrigerator for about 3 months [2]. The hemagglutination activity of lectin-2 is insensitive to lactose, but is sensitive to a number of saccharides, including *N*-acetyl-D-galactosamine [2].

A major impediment to work with lectin-2 was the lack of a reliable method for preparing erythrocytes that were agglutinated by it. This report describes methods for preparing such erythrocytes. It also describes the inhibition of lectin-2 activity by low concentrations of specific glycosaminoglycans, raising the possibility that the lectin might play a role in interaction of developing muscle cells with components of the extracellular matrix.

2. Materials and methods

2.1. *Preparation of type I erythrocytes*

Trypsin-treated, glutaraldehyde-fixed rabbit erythrocytes, prepared by methods similar to those in [3], are referred to as type I erythrocytes. They were stored at 6°C as a 10% suspension in 75 mM NaCl, 75 mM Na_2HPO_4/KH_2PO_4 (pH 7.2) (PBS) for ≤ 4 weeks. Prior to the assay they were diluted with 0.1 M glycine in PBS to produce a 4% suspension.

2.2. *Preparation of type II erythrocytes*

Type II erythrocytes were prepared by transformation of type I erythrocytes by incubation under various conditions or by ethanol treatment as described in the text. After transformation they were stored as a 10% suspension (see table 2). Prior to assay they were diluted with 0.1 M glycine in PBS to produce a 4% suspension.

2.3. *Hemagglutination activity*

This was determined using serial 2-fold dilutions of extract in microtiter V-plates (Cooke Engineering). Each well contained 25 μl erythrocyte suspension, 25 μl extract diluted in PBS containing 4 mM β-mercaptoethanol, 2 mM ethylenediaminetetraacetic acid (MEPBS), 25 μl 1% bovine serum albumin in 0.15 M NaCl and 25 μl 0.15 M NaCl. To study the effects of inhibitors of hemagglutination, appropriate concentrations of inhibitor in 0.15 M NaCl were added to the well in 25 μl aliquots, replacing the equivalent volume of saline. Titers were determined 1 h later. The titer is the highest dilution of extract that agglutinated the cells (e.g., 1:256). Specific activity is titer^{-1} (e.g., 256) divided by mg protein/ml extract. Protein was determined by the method in [9].

2.4. *Preparation of extracts*

These were prepared as described in table 1.

2.5. *Materials*

Heparin, heparan sulfate and dermatan sulfate were generous gifts of Dr A. Linker, Univ. Utah, Salt Lake City. Hyaluronic acid, chondroitin-4-sulfate and chondroitin-6-sulfate were obtained from Sigma. Saccharides were obtained from Pfantstiehl or Sigma.

3. Results

3.1. *Separation of lectin-1 from lectin-2 by centrifugation*

As a first step in the characterization of lectin-2, it was necessary to find a reliable method for preparation of erythrocytes that were agglutinated by it. This, in turn, required a preparation of lectin-2 devoid of lectin-1 activity. We found that upon centrifugation of extracts of embryonic chick muscle at 100 000 × *g* for 12 h, all the lectin-1 activity remained in the supernatant whereas almost all of the recovered lectin-2 activity was found in a small pellet at the bottom of the centrifuge tube (table 1). This partially purified lectin-2 was used for further studies.

Table 1
Separation of lectin-1 and -2 by high speed centrifugation

	Titer^{-1}	
	Type I erythrocytes	Type II erytheocytes
Soluble extract	256	1024
Supernatant (100 000 × *g*, 12 h)	256	32
Pellet (100 000 × *g*, 12 h)	8	512

Pectoral muscle from day 16 chick embryos was homogenized for 1 min in a Sorvall Omni-Mixer in 9 vol. MEPBS containing 0.1 M lactose and 0.1 M *N*-acetyl-D-galactosamine and centrifuged for 1 h at 100 000 × *g*. The supernatant (designated soluble extract) was then centrifuged for 12 h at 100 000 × *g* giving rise to a supernatant and a pellet. The latter was resuspended in a volume of MEPBS equal to that of the supernatant. Lectin activity was so high that the sugars in the homogenizing medium were diluted to non-inhibiting concentrations in the serial dilutions of the assay. The type II erythrocytes used here had been transformed from type I erythrocytes by storage in the refrigerator for 4 months. Hemagglutination activity with type I erythrocytes was inhibited > 95% by 10 mM lactose and was not affected by 10 mM *N*-acetyl-D-galactosamine. Hemagglutination activity with type II erythrocytes was inhibited 50% by 10 mM *N*-acetyl-D-galactosamine and was not affected by 10 mM lactose

3.2. *Preparation and properties of type II erythrocytes*

Our initial preparations of type II erythrocytes were spontaneously transformed from type I erythrocytes upon storage in the refrigerator for several months [2]. By trial and error we found that higher temperatures accelerated this transformation and made it much more reliable (table 2). We have no explanation for this. Since incubation at 60°C was most effective (table 2), it is very unlikely that the transformation is due to the action of bacterial contaminants or to residual enzyme activity in the fixed erythrocytes. In the course of these studies we also found that treatment with ethanol is a rapid and reliable method for transforming type I erythrocytes (table 2). In subsequent studies we used type II erythrocytes prepared by ethanol treatment as described in table 2.

In an attempt to identify a difference between type I and type II erythrocytes, we sought to determine their relative reactivity with other lectins. In general, type II erythrocytes were less agglutinable (table 3). This suggests that the difference is related to specific binding sites that are displayed on the type II erythrocytes rather than to a general change in agglutinability. Display of such 'type II receptors' could be determined by many factors which control their organization in the membrane.

3.3. *Lectin-2 inhibition by glycosaminoglycans*

Several glycosaminoglycans are potent inhibitors of lectin-2 activity (table 4). Heparin and dermatan sulfate were consistently most potent. At 5-fold higher concentrations than those in table 4 they inhibited ⩾ 87.5% of lectin activity. Heparan sulfate was a weaker inhibitor and hyaluronic acid, chondroitin-4-sulfate and chondroitin-6-sulfate had no detectable effect at the highest concentrations that could be used without interfering with the assay. Assuming mol. wt ~2 × 10^4 for heparin, ~10^{-4} mM produces 50% inhibition. The most potent simple saccharide studied, *N*-acetyl-D-galactosamine produced 50% inhibition at molar concentrations almost 5 orders of magnitude higher than heparin.

Table 2
Rate of transformation of type I to type II erythrocytes by different treatments

Treatment	Titer^{-1} at the indicated weeks of incubation							
	0.1	0.5	1	2	3	4	12	16
6°C	0	0	0	0	0	0	512	512
22°C	0	0	0	0	0	512	512	512
37°C	0	0	0	512	512	512	512	512
60°C	0	256	512	512	512	512	512	512
Ethanol	512	512	512	512	512	512	512	512

Preparation of type II cells by incubation at different temperatures: Aliquots of type I cells were stored as a 10% suspension in PBS at the indicated temperature until the observed titer had stabilized. Thereafter they were stored at 6°C. They were assayed, after the indicated periods of incubation with aliquots of lectin-2 that had been pelleted, as in table 1, resuspended in MEPBS to the initial extract volume, and kept frozen in aliquots. A separate aliquot was thawed for testing at each time point. Preparation of ethanol-treated type II cells: Type I cells were centrifuged at 1000 × *g* for 5 min and the pellet was resuspended in 9 vol. 100% ethanol and gently shaken for 1 h at room temperature. The cells were centrifuged, resuspended in 100% ethanol and again shaken for 1 h. The cells were then washed 5 times in PBS, and stored as a 10% suspension in PBS at room temperature. Titer with lectin-2 was maximal within a day of treatment but the wells containing relatively high concentrations of lectin (e.g., dilutions of 1/2 to 1/16) showed no agglutination and only the lower concentrations (1/32 to 1/512) showed agglutination. After several days this 'high concentration inhibition' was no longer seen

Table 3
Relative agglutination of type I and type II erythrocytes by various lectins

Lectin	Titer^{-1}		
	Type I erythrocytes	Type II erythrocytes (37°C)	Type II erythrocytes (ethanol)
Muscle lectin-1	256	64	4
Muscle lectin-2	0	256	256
Concanavalin A	256	128	32
Wheatgerm agglutinin	256	64	256
Lentil agglutinin	256	64	16
Soybean agglutinin	256	64	16

A concentration of lectin that gave a titer of 1/256 with one of the cell preparations was determined and the titer with the other cells was determined using the identical series of lectin dilutions. Lectin-1 was purified by the method in [3]; lectin-2 was obtained by centrifugation for 12 h as in table 1. The other lectins were from commercial sources. Type II erythrocytes were prepared by incubation of type I erythrocytes at 37°C or by treatment with ethanol, as described in table 2

Table 4
Inhibition of hemagglutination activity of lectin-2 by glycosaminoglycans and other saccharides

Saccharide	Concentration (μg/ml) inhibiting 50% of lectin activity
Heparin	2
Dermatan sulfate	4
Heparan sulfate	12
Chondroitin-4-sulfate	> 50
Chondroitin-6-sulfate	> 50
Hyaluronic acid	> 50
N-acetyl-D-galactosamine	1000
Cellobiose	2000
N-acetyl-D-glucosamine	> 15 000

3.4. *Lectin-2 in other embryonic chick tissues*

As with lectin-1 [5,6] we found lectin-2 activity in heart, liver and brain of day 16 chick embryos. The specific activity of the heart extracts is similar to that of pectoral muscle; it is lower in the other tissues.

4. Discussion

We have shown [1,2] that embryonic muscle contains two lectin activities that change with development. A limiting factor in studies of lectin-2 was the lack of a reliable method for preparing test erythrocytes. Our finding that treatment of trypsinized, glutaraldehyde-fixed rabbit erythrocytes with ethanol produces reliable test cells will facilitate further work. Whereas the nature of the transformation by ethanol is not known, it should be pointed out that the mechanism of generation of test cells that are reactive with some other lectins (e.g., by trypsinization) is equally obscure. Some possible modifications which may sensitize test cells include the exposure of latent receptors and the reorganization of certain membrane constituents so that receptors are in a more agglutinable form.

Lectin-2 is an unusual lectin not only because it requires unusual test cells but also because it is present in tissue extracts in a high molecular weight form, presumably some type of aggregate. This property has permitted its initial separation from crude extracts by high speed centrifugation. However, complete purification has not yet been achieved, because of marked activity losses with many of the procedures that we used.

Another unusual property of lectin-2 is that its hemagglutination activity is markedly inhibited by specific glycosaminoglycans. Lectin-1 activity is not inhibited by these substances. It remains to be determined if inhibition of lectin-2 activity is due to interaction of the glycosaminoglycans with its carbohydrate binding site. Inhibition does not appear to be a nonspecific effect of polyanions since other glycosaminoglycans have no detectable effect. It is notable that all the inhibitory glycosaminoglycans contain some iduronic acid. The presence of iduronic acid residues in glycosaminoglycans has been shown [10] to correlate with their binding to many proteins. However, interactions between glycosaminoglycans and proteins can be highly specific [10]. The specific hexosamine in the glycosaminoglycans does not appear critical for inhibitory activity. *N*-acetyl-D-galactosamine, an inhibitory saccharide, is present in dermatan sulfate an inhibitor; but also in chondroitin sulfate and hyaluronic acid. Heparin and heparan sulfate, other potent inhibitors, contain *N*-acetyl-D-galactosamine.

As far as we can determine lectin-2 bears no relationship to any known protein that interacts with glycosaminoglycans. One protein which interacts with heparin is fibronectin [11], an extracellular protein [12] which has been shown to have hemagglutination activity [13]. One difference between lectin-2 and fibronectin is that the hemagglutination activity of fibronectin is markedly inhibited by low concentrations of EDTA [12]; but this is routinely present in relatively high concentrations in our assay of lectin-2. Fibronectin is also absent from preparations of lectin-2 that we partially purified by adsorption to a cellulose column, elution with alkali, and fractionation on Sepharose-6B. The product is only purified ~20-fold and reveals several protein bands after SDS–polyacrylamide gel electrophoresis under conditions capable of resolving fibronectin; but the largest has mol. wt 42×10^3, far smaller than fibronectin. The possible relationship of lectin-2 to a cell surface substance that binds heparin and related glycosaminoglycans [14], as well as its function in developing tissues, remains to be determined.

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