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Detection of four types of specific Phytohemagglutinins in different lines of beans (Phaseolus vulgaris)

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Abstract

When the hemagglutinating action of extracts from ground seeds of different lines or cultivars of beans (Phaseolus vulgaris) was tested with blood cells from different animals, four different patterns of activity were distinguished. The most common type, called A, agglutinated all blood types tested with the exception of cow blood cells which were rendered susceptible to agglutination by treatment with trypsin. Type-B beans were active on all the blood types except trypsin-treated cow blood, type-C bean extracts agglutinated strongly only trypsin-treated cow blood cells and pronase-treated rat and hamster cells. The last type, called D, had little if any activity with the exception of its action on pronase-treated hamster blood cells.

The hemagglutinating activity on trypsin-treated cow blood cells of A- or C-type extracts was not destroyed after 90 minutes heating at 80° C but the activity on rabbit blood cells was lost.

The hemagglutinating activity of extracts of any one of these four bean types could be absorbed on rabbit or trypsin-treated cow blood cells and released again by heating to 56° C although some of the extracts would not agglutinate these types of blood cells. The disappearance of at least one component of the bean extracts after repeated absorption with blood cells was detected by immunoelectrophoresis. This component was detected in the supernatant after release from the cells. The corresponding immunoprecipitation line could be stained with sudan black indicating that it was caused by a lipoprotein.

The possibility that the A-type activity is caused by a combination of B- and C-type hemagglutinins is discussed. The results of a cross-breeding experiment which revealed the fact that this activity is inherited as a single, dominant trait is evidence against this possibility.

The use of trypsin-treated cow blood cells and rabbit rbc for testing different fractions from bean extracts in recommended.

The existence of phytohemagglutinins (PHA) in the seeds of kidney beans (Phaseolus vulgaris) has been known since Landsteiner and Raubitschek's first observation in 1908 (9). These authors not only discovered hemagglutinating activity in the extracts of seeds from different legumes but pointed out also that many of these extracts act only on the red cells from some animal species and not on those from others. Kidney bean extracts, however, were active with all the blood

[1]) deceased

samples. Indeed, when red blood cells from 32 different animal species were tested with bean extracts in our laboratory, agglutination activity was observed in all cases except with cow blood cells which had to be treated with trypsin to make them sensitive to the agglutinating action (7). In some samples the hemagglutinating principle could not be found because the extracts of the seeds of some strains of kidney beans (Phaseolus vulgaris) and of wild beans (Phaseolus aborigineus) did not agglutinate the erythrocytes tested (3).

The recent interest in kidney bean PHA is due to its capacity to stimulate tissues culture lymphocytes to undergo mitosis. It has become a potent tool for the study of a wide variety of biological phenomena related to this process. Information about the reason for the apparent lack of hemagglutinating and mitogenic activity in some bean cultivars may be of importance in the work on chemical definition of the factor or factors responsible for these activities.

In this paper we present experimental evidence that by use of some enzyme activated erythrocyte preparations hemagglutinating activity can be detected also in bean varieties considered until now as inactive, and that four different hemagglutinin specifities can be distinguished in different beans by this way.

Materials and Methods

Most of the seeds tested were harvested from genetically pure lines of beans which had been cultivated under controlled conditions in our experimental fields. Crosses of bean plants were performed by artificial pollinization for the genetic analysis.

The extracts were prepared from seeds ground in a Wiley Laboratory mill. The ground material was mixed with ten times the amount of physiological saline for 2 hours and clarified by centrifugation.

Red blood cells (rbc) were separated from citrate-containing samples by centrifugation and twice washed with physiological saline. The red cells from 10 ml of cow blood were suspended in 10 ml of saline, 0.1 mg of crystalline trypsin added, and after one hour standing at 25° spun down and twice washed with saline. Pronase (Calbiochem, Los Angeles, Calif.) was used in the proportion of 1.0 mg/10 ml of 4% erythrocyte suspension. After 60 minutes digestion time at 25° the blood cells were centrifuged and twice washed with saline solution. The hemagglutination tests were performed in serial dilutions with a microtiter kit (Cooke Eng. Comp. Alexander, Virginia) and read after one hour standing at room temperature. Only 12 dilution steps were tested. The 12th dilution step of a bean extract corresponds to a dilution of about 1 : 500000 of the extracted proteins.

Rabbit immunosera were prepared as described earlier (10) using a saline extract of different bean cultivars as antigen. Immunoelectrophoresis was performed by the micro-technique (5) using veronal buffer *p*H 8.2 and the preparations were stained with azocarmin or sudan black, the latter for the detection of lipoproteins. In most cases the immunosera were fractionated with Na_2SO_4 before use (4).

Absorption experiments were performed by suspending washed and packed erythrocytes in the corresponding bean extracts, mechanical agitation for 30

minutes, separation of the agglutinated cells by centrifugation, and repeating the same process four times. For eluation of the absorbed material the agglutinated cells from the first two treatments were twice washed with physiological saline, suspended in saline adjusted to *p*H 5, heated to 56° for 10 minutes and centrifuged. Determination of hemagglutinating activity and immunoelectrophoretic analysis was performed with the bean extract after each absorption and with the eluates.

Results

The dilution steps of extracts from selected samples of ground beans capable of agglutinating rbc of different animals or humans of different blood groups are presented in Table 1. Only one example of each of the four different patterns which have been detected is included in the table. The first type of specifity, called A, agglutinated all the blood samples tested with the exception of untreated cow erythrocytes. Trypsin-treated cow red cells, however, were agglutinated in high dilution. The second type, called B, was also active on most types of blood but, contrary to the A-type, the activity toward trypsin-treated cow rbc was weak or absent. The third type of beans, called C, showed little or no activity in contact with any one of the native red blood cells tested but agglutinated trypsin-treated cow rbc and pronase

Tab. 1. Hemagglutinating activity of extracts of four types of beans on different blood samples*)

Type of blood	Bean cultivar							
	Saxa (A)		Cubagua (B)		Porillo (C)		Mountan. Half Runner (D)	
	nat.**)	pron.**)	nat.	pron.	nat.	pron.	nat.	pron.
Rabbit	8	11	9	11	0	2	0	2
Cow***)	0	12	0	2	0	12	0	1
Swine	11	12	9	12	0	0	0	0
Mouse	6	12	3	8	3	7	1	5
Rat	6	11	2	8	0	9	0	4
Hamster	8	12	7	11	6	12	3	7
Cat	12	12	5	12	0	5	0	4
Cock	6	12	6	11	0	9	0	3
Sheep	4	12	3	6	2	7	0	1
Human blood group AB	6	12	6	12	0	4	0	3
Human blood group 0	6	12	6	12	0	3	0	3

*) The highest dilution step of the crude seed extracts (1 g of ground seeds + 10 ml physiological saline) producing visible agglutination within one hour is indicated. Only 12 dilution steps were tested.

**) The first column refers to native rbc, the second to pronase treated rbc.

***) Cow rbc were treated with trypsin, instead of pronase.

treated rat and hamster rbc very efficiently. The last type of extracts, called D, had very little if any activity on native blood cells of any kind, but was able to agglutinate efficiently pronase-treated hamster cells. Treatment with pronase enhanced the susceptibility to agglutination of most blood cells as has been observed by Pardoe et al. (11).

For further tests to detect or differentiate these four types of bean agglutinins, rabbit and trypsin-treated cow rbc were used because all four specificity types could be distinguished as can be seen from some representative results presented in Table 2. Single seeds from some 155 different cultivars and sublines of beans were tested. Beans able to agglutinate rabbit and trypsin-treated cow rbc (A-activity) are the most common and have numerous representatives among commercial cultivars. Activity toward rabbit blood only (B-activity) was found in relatively few strains of so called «Tropical beans». These represent a filetic line of small seeded, semirunner varieties with neutral photoperiodic response (2). Some cultivars are mixed populations of A- and B-active seeds. Extracts active in agglutinating trypsin-treated cow rbc only (C-activity) are rare and have been found in some indigenous cultivars of black and red pole beans from Ecuador to Central America and including a few commercial varieties. Seeds without significant activity toward rabbit and cow blood (D-activity) are very rare in the indigenous beans but are found in some commercial varieties like Mountaneer Half Runner, Sword, Alabaster, Kaiser Wilhelm.

There exists a notable difference in heat-resistance as can be seen from the data included in Table 2. Ninety minutes heating at 80° C

Tab. 2. Hemagglutinating activity of extracts of different bean cultivars to rabbit and trypsin-treated cow blood cells before and after heating at 80° C for 90 minutes

Bean cultivar	Agglutination titer before heating		after heating	
	rabbit rbc	cow rbc	rabbit rbc	cow rbc
Saxa (A)	9	11	4	11
Balinde albenga (A)	9	10	5	10
Guateian (A)	11	9	2	9
Juli (B)	8	3	1	0
Cubagua subline (B)	9	2	0	0
San Fernando (B)	8	3	0	0
Porillo (C)	0	9	0	9
Ecuador 4A (C)	0	11	0	11
Rabuda (C)	0	9	0	9
Mountaneer (D) half runner	0	0	0	0
Great northern (D)	0	0	0	0
Kaiser Wilhelm (D)	0	0	0	0

The highest dilution step producing visible agglutination in one hour is indicated. The extracts were heated in the presence of the ground beans used for extraction.

destroys completely or almost completely the activity detected with rabbit rbc but not that observed with trypsin-treated cow rbc. The heat-lability of the B-type bean extracts can be used as an additional criterion to distinguish them from A-type extracts.

When an extract of any one of the four types of beans was repeatedly absorbed with rabbit rbc or trypsin-treated cow rbc hemagglutinating activity disappeared. The action on rabbit rbc disappeared more rapidly from an A-type extract than the activity on cow rbc. The active material thus absorbed could be brought into solution again by heating the red blood cells at 56° C for 10 minutes and followed by centrifugation. The hemagglutinating activity was found in the supernatant.

Immunoelectrophoresis of the crude bean extracts reveals the formation of at least 8 different precipitation lines, two of which can be stained with sudan black. They are both located toward the cathode. One of these lines disappears in the supernatant after absorption with red cells. The eluates liberated by heating the rbc used for absorption were tested by immunoelectrophoresis with each of the four antisera produced by the injection of A, B, C, or D-type bean extracts into rabbits. At least one cathodic precipitation was always observed and in most cases two additional precipitation lines were discernible. One of these moved toward the cathode and one toward the anode. The immunochemical reactivity of the material in the eluate from a C or D-type bean extract and the antiserum produced with A-type extract was less clear than that observed when antigen and antiserum were homologous but a precipitate was always discernible at the cathodic side of the immunoelectrophoretic slide.

The inheritance distributions as revealed by the F_2-crosses of A- and D-type bean lines are presented in Table 3. No segregation between

Tab. 3. Hemagglutinating activity in the F_2-generation of crosses of type A and D bean plants

No.	Type of cross	Agglutination of rabbit and trypsin-treated cow rbc Number of seeds	
		+	—
1	A/D	23	13
2	A/D	76	28
3	A/D	22	14
4	D/A	30	6
5	D/A	55	22
6	D/A	241	81
7	D/A	231	77
		678 (73.7%)	241 (26.3%)

Extracts from each seed were tested with rabbit and trypsin-treated cow blood cells separately. All positive seeds showed activity with both blood types.

the specific hemagglutinating activities was detected in 919 single seeds tested with both rabbit and trypsin-treated cow rbc. All were active on both kinds of blood cells or devoid of any of these activities.

Discussion

The existence of at least four distinctly different hemagglutinins in different bean seeds has been demonstrated by the results presented in Tables 1 and 2. In previous work on bean PHA only hemagglutinin-containing and non-hemagglutinating beans could be distinguished because trypsin-treated cow blood cells had never been used for screening although they had been known to be agglutinated by bean extracts (7). We could not detect a difference between the agglutination titers of human AB and 0 blood group samples as had been observed for some bean extracts by Toms and Turner (14) (Table 1). It is noteworthy that trypsin-treatment sensitized cow rbc to agglutination but pronase-treatment produced minimal sensitization or none at all. Furthermore, pronase did not diminish the agglutination of trypsin-treated cow erythrocytes and, therefore, probably did not act on the receptor sites. Pardoe et al. (11), however, have observed some activation of cow rbc with pronase.

The activity of the A-type bean extracts could be considered as the sum of the B-type and the C-type activities both in specific agglutination and with respect to the heat-resistance of these specific agglutination activities. The exception to this observation was the activity toward cat blood which was much stronger in the A-type beans than in the B- and C-type beans together. The C-type and D-type bean agglutinins behaved like «incomplete» agglutinins because they required the specific blood cells to be submitted to proteolytic activation in order to be agglutinated by them (Table 1).

The observation that the capacity to agglutinate rabbit rbc of an A-type bean extract absorbed with red cells disappears more readily than its activity toward trypsin-treated cow rbc can probably be explained by the greater sensitivity of the latter reaction and should not be taken as evidence that two different factors are responsible. The difference in the heat-sensitivity of the two reactions, however, would point to the involvement of two different factors or to the existence of two active centers in one molecule which should behave differently when heated.

Two well separated active elution peaks can be obtained by chromatography on DEAE-cellulose of an extract of a genetically pure A-type bean sample. Both peaks show A-type activity and we could not achieve separation of fractions with hemagglutinating action toward rabbit and cow rbc.

The cross-breeding experiments were performed as still another approach to the problem whether the A-type activity is due to a single factor or to multiple factors. The results presented in Table 3 are consistent with the existence of a single dominant factor responsible for the inheritance of the activity to agglutinate both types of blood.

Rabbit rbc and trypsin-treated cow rbc are able to take up all four types of bean hemagglutinins from the respective seed extracts although agglutination is not produced in all cases as shown in Table 1. STECK and WALLACH (13) obtained similar results in their work on bean agglutinin absorbed on different cell types and concluded that agglutination and absorption are related but discrete phenomena.

In previously published studies we had observed that in the immuno-precipitations formed from rabbit blood hemagglutinating bean extract and homologous antisera at least one line could be stained with sudan black but that the non-hemagglutinating extracts did not produce a precipitation which could be stained in this way (10). This is contrary to the present observation that the material liberated from rabbit erythrocytes after absorption of any one of the four bean types could be thus stained. The reason for these different results is probably due to quantitative differences in stainable material in the different bean extracts. All four bean agglutinins may be lipoproteins as has been shown to be the case with other phytohemagglutinins (8).

In further experiments we observed that only the A and C-type agglutinins i.e. those active on trypsin-activated cow rbc show mitogenic activity on cultured human lymphocytes; these results will be described in detail in a separate publication. Several authors have claimed that a hemagglutinating factor from bean extracts can be separated from a mitogenic factor devoid of hemagglutinating action (1, 6, 12, 15). In these experiments only human or rabbit blood was used for testing the hemagglutinating activity. It is suggested that trypsin-treated cow blood and, in special cases, also pronase-treated hamster blood be used for the hemagglutinating assays in order to detect the C- and D-type bean agglutinins described in this paper.

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Zusammenfassung

Nachweis von 4 spezifischen Phythaemagglutinintypen bei verschiedenen Bohnenlinien (Phaseolus vulgaris)

Vier verschiedene Arten von Haemagglutininen konnten in verschiedenen Linien von Gartenbohnen (Phaseolus vulgaris) nachgewiesen werden mit Hilfe ihrer unterschiedlichen Fähigkeit, verschiedene Arten von roten Blut-

körpern zu agglutinieren. Der häufigste Agglutinin-Typ wurde «A» genannt; er agglutiniert alle untersuchten Blutsorten mit Ausnahme von Kuhblut, das jedoch nach Trypsin-Behandlung auch agglutinationsempfindlich wird. Bohnenextrakte des B-Types agglutinierten alle untersuchten Blutsorten mit Ausnahme von trypsin-aktiviertem Kuhblut; Extrakte von Samen des C-Types agglutinieren nur trypsin-aktivierte Kuhblutzellen und pronase-aktivierte Hamsterblutzellen, während die Samen des D-Types Extrakte liefern, die nur mit pronase-aktiviertem Hamsterblut wesentliche Aktivität zeigen.

Während die Agglutininaktivität gegen trypsin-aktiviertes Kuhblut nach Erhitzen auf 80° C für 90 Minuten praktisch unverändert blieb, war die Agglutinationswirkung auf Kaninchenblut nach dieser Behandlung praktisch verschwunden.

Durch Behandlung mit Kaninchen- oder trypsin-aktivierten Kuhblutzellen konnten alle 4 Bohnenagglutinine absorbiert werden und durch Erhitzen der zur Absorption benützten Blutzellen auf 56° C wieder freigesetzt werden. Durch Immunoelektrophorese konnte das Verschwinden von wenigstens einem Antigen aus den Rohextrakten durch die Absorption und dessen Freisetzung durch Erwärmen auf 56° C beobachtet werden. Die entsprechende Immunoprezipitationslinie wurde durch die Sudanschwarzfärbung als Lipoprotein erkannt.

Es wird die Frage erörtert, ob die A-Aktivität durch eine Kombination von B- und C-Agglutininen erklärt werden kann. Dazu wird u. a. ein Kreuzungsversuch herangezogen, der ergab, daß erstere wie eine einzige, dominante Erbeigenschaft vererbt wird. Die Verwendung von Kaninchenblut- und trypsin-aktivierten Kuhblutzellen wird für Versuche über die Trennung von Agglutininen und mitogenen Faktoren in Bohnenextrakten empfohlen.

Résumé

Détection de quatre types de phytohémagglutinines spécifiques dans différentes séries de fèves (Phaseolus vulgaris)

Quatre types différents d'hémagglutinines ont pu être détectés dans diverses séries de fèves (Phaseolus vulgaris) à l'aide de leur capacité variée d'agglutiner différentes sortes de globules rouges. Le type d'agglutinine la plus fréquente a été appelé «A»: elle agglutine toutes les sortes de sang examinées, à l'exception du sang de vache; mais ce dernier devient également sensible à l'agglutination après traitement avec la trypsine. Des extraits de fèves du type B agglutinèrent toutes les sortes de sang examinées, à l'exception du sang de vache, activé à la trypsine; les extraits de semence du type C ont agglutiné seulement les cellules sanguines de vache, activées à la trypsine, et celles du hamster activées à la pronase, tandis que les semences du type D produisent des extraits, qui présentent une activité notable seulement dans le sang de hamster, activé à la pronase.

Tandis que l'activité de l'agglutinine reste pratiquement inchangée après avoir été échauffée à une température de 80° C pendant 90 minutes, l'effet de l'agglutination sur le sang de lapin avait disparu.

En les traitant avec du sang de lapin ou des cellules de sang de vache activées à la trypsine, toutes les 4 agglutinines d'haricot purent être absorbées et de nouveau être libérées des cellules sanguines utilisées pour l'absorption en les échauffant à 56° C. Au moyen de l'immuno-électrophorèse, il était possible d'observer la disparition au moins d'un antigène des extraits bruts par l'absorption et de son dégagement en échauffant à 56° C. La ligne d'immunoprécipitation fut reconnue par la coloration noire Soudan en tant que lipoprotéine.

On a discuté pour savoir si l'activité A peut s'expliquer par une combinaison des agglutinines B et C. Entre autres, on a tenté un essai de croisement, qui démontra que l'activité A est héritée comme une seule caractéristique hérédi-

taire dominante. L'emploi de sang de lapin et des cellules de sang de vache activées à la trypsine est recommandé pour les essais de séparation des agglutinines et des facteurs mitogènes dans les extraits d'haricot.

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