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Glutamate Oxaloacetate Transaminase Isozymes of the *Triticinae*: Dissociation and Recombination of Subunits*

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Summary. A simple procedure has been developed for the dissociation of active molecules of glutamate oxaloacetate transaminase (GOT: E.C. 2.6.1.1) into protomers and for the reassociation of the subunits into active enzymes. Results of experiments in which the protomers of genetically controlled electrophoretic variants of GOT of *Triticum aestivum* and of several related species were dissociated and recombined in crude tissue extracts and in partially purified preparations support the hypothesis that the enzyme exists functionally as a dimer in the Triticinae.

Key words: Glutamate Oxaloacetate Transaminase - Isozymes - *Triticum aestivum* - Subunits

Introduction

Genetic evidence that glutamate oxaloacetate transaminase (L-aspartate: 2-oxoglutarate aminotransferase, E.C. 2.6.1.1; GOT; also known as aspartate aminotransferase) exists functionally as a dimer has been obtained in studies of several higher plant species, including *Zea mays* (MacDonald and Brewbaker 1972; Scandalios et al. 1975), *Stephanomeria exigua* (Gottlieb 1973), and *Triticum aestivum* and the related species *Secale cereale* and *Agropyron elongatum* (Hart 1975; Tang and Hart 1975; Hart et al. 1976). We now report experiments in which the subunits of genetically determined electrophoretic variants of the GOT-3 system of *T. aestivum* and of several related species have been dissociated and recombined in vitro. The results of these experiments support the hypothesis of a dimeric structure for the active GOT isozymes. A preliminary report of this research has been published elsewhere (Hart and Langston 1976).

Hexaploid wheat (*T. aestivum*, 2n = 42, genomes A, B, and D) expresses several genetically independent GOT systems. The GOT-3 system is composed of three isozymes. A zymogram analysis of appropriate aneuploid derivatives of the cultivar 'Chinese Spring' provided evidence that these isozymes are the products of a triplicate set of structural genes located in the chromosomes of homoeologous group 3. The results of the analysis were consistent with the proposal that three structural genes, designated Got-A3, Got-B3, and Got-D3, located in chromosome arms 3AL (formerly 3Aα), 3BL and 3DL (formerly 3Dα), respectively, encode approximately equal quantities of three subunits, designated α^3, β^3, and δ^3, respectively, which randomly dimerize to produce six approximately equally active forms. The designations GOT-3a, GOT-3b, and GOT-3c have been assigned to the GOT-3 isozymes which migrate at relatively fast, intermediate, and slow rates, respectively, during electrophoresis at a slightly alkaline pH. It is supposed that GOT-3a is composed of $\beta^3\beta^3$, $\delta^3\delta^3$, and $\beta^3\delta^3$ dimers, GOT-3b of $\alpha^3\beta^3$ and $\alpha^3\delta^3$ dimers, and GOT-3c of $\alpha^3\alpha^3$ dimers (Hart 1975).

Additional genetic evidence that GOT exists functionally as a dimer was obtained in two other studies involving *T. aestivum*. Tang and Hart (1975) determined the zymogram phenotype of each member of the series of seven disomic *Secale cereale* (2n = 14, genome R) chromosome additions to *T. aestivum* and of the *T. aestivum-S. cereale* amphiploid. Their analysis provided evidence that chromosome 3R possesses a structural gene, Got-3R, whose product, ρ^3, ran-

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domly dimerizes with the products of the *T. aestivum* Got-3 set. Examination by Hart et al. (1976) of a series of *T. aestivum-Agropyron elongatum* translocation lines, in each of which a segment of the long arm of chromosome 3D has been replaced by a portion of the *A. elongatum* homoeologue 3Ag, provided evidence that the products of the Got-3 set of hexaploid wheat randomly dimerize with ε^3, the product of Got-3Ag, a homoeologous gene located in 3AgL.

Exposure of different isozymes of a GOT system to in vitro conditions in which dissociation of the isozymes into protomers and reassociation of the subunits into active enzymes occurs critically tests the hypothesis that the active forms of GOT are dimers. Support for the hypothesis would come, for example, from the demonstration that dissociation and recombination of the subunits of two different supposedly homodimeric isozymes results not only in the regeneration of the original two isozymes but also in the production of a form with the properties expected of a heterodimer. Support would also come from the production of two forms with the properties of homodimers, in addition to the original enzyme, from a supposedly heterodimeric enzyme.

The several experiments which are the subject of this report involve dissociation and recombination of the subunits of GOT-3 forms which differ in electrophoretic mobility. The strains used each express either one or three GOT-3 isozymes. Individual isozymes were obtained from strains expressing three GOT-3's by elution from starch gels after electrophoresis of crude tissue extracts. Experiments which utilized strains that express but one GOT-3 enzyme were performed with crude extracts.

Materials and Methods

Strain G919 of *T. monococcum* (2n = 14, genome A), the cultivar 'Imperial' of *S. cereale* (2n = 14, genome R), a nullisomic-3A tetrasomic-3D derivative of the cultivar 'Chinese Spring' of *T. aestivum* (2n = 42, genomes A, B, and D), the cultivar 'Rolette' of the durum group of *T. turgidum* (2n = 28, genomes A and B), the 'Chinese Spring'-'Imperial' amphiploid (2n = 56, genomes A, B, D, and R) and a *T. aestivum-A. elongatum* chromosome addition line (2n = 44, genomes A, B, and D plus 1" 3Ag) were utilized in this study. The first three strains listed each express one form of GOT-3 and the last three strains each express three GOT-3 isozymes.

The nulli-3A tetra-3D strain expresses GOT-3a while the *T. monococcum* and *S. cereale* strains each express a GOT-3 which has an electrophoretic mobility coincident with that of GOT-3c of *T. aestivum*. Recombination of the subunits of GOT-3a of the nulli-3A tetra-3D strain with the GOT-3 subunits of each of the two other strains is accomplished by the following simple procedure. Appropriate tissue containing GOT-3 (Hart 1975) obtained from each of the two selected strains is macerated in separate mortars with pestles in a 0.1 M potassium phosphate buffer, pH 6.0, containing 40 m moles pyridoxal 5' phosphate. The slurry obtained from each maceration is centrifuged at 30,000 × g for 20 min, after which aliquots of the two supernatants, of appropriate quantities so that they contain approximately equal amounts of GOT-3 activity, are mixed and incubated for 2 hours. All operations are carried out at 2-5°C. Following the 2 hour incubation, the mixture is made to 0.1 M sucrose and then electrophoresed at an alkaline pH in disc polyacrylamide gels, at the completion of which the gels are stained for GOT activity. The methods used for electrophoresis and GOT staining have been described previously (Hart 1975).

The source of the GOT-3 enzymes for the experiments reported here were scutella obtained from mature grains that had been germinated in moist paper toweling for 18 hours. A 1:100 ratio (w/v) of scutella to buffer was used. Dissociation and recombination of GOT-3 subunits in crude tissue extracts has also been accomplished using the first foliage leaf and the coleoptile as the source of the GOT-3 enzymes.

Three types of reference samples were routinely prepared and included in these experiments. One consisted of a mixture prepared as described above, except for the use of a pH 7.0 rather than a pH 6.0 phosphate buffer. The second consisted of extracts of the two selected strains which were individually subjected to the pH 6.0 procedure, without mixing of the extracts. The third consisted of extracts of the two selected strains which were prepared immediately prior to electrophoresis by the method used in the earlier genetic studies of the Triticinae GOT's (Hart 1975). It should be noted that the relative electrophoretic mobilities of GOT-3 enzymes contained in different gels were easily determined because in all gels isozymes of other genetically independant GOT systems were also expressed.

The *T. turgidum* strain, the *T. aestivum-S. cereale* amphiploid, and the *T. aestivum-A. elongatum* chromosome addition line each express three GOT-3 isozymes, the electrophoretic mobilities of which are coincident with those of GOT-3a, -3b, and -3c of *T. aestivum*. The isozymes used in the subunit recombination experiments conducted with these strains consisted in each case of partially purified preparations of the electrophoretically fast and slow forms that were obtained by elution from starch gels after electrophoresis of a crude scutellar extract. Elution of the isozymes and recombination of the subunits is accomplished by the following procedure. A concentrated extract is prepared by maceration of tissue in a mortar with pestle in a pH 7.5 buffer described by Carlson (1972), also containing 0.1 M sucrose. The scutella used for the experiments reported here were obtained from mature grains that had been germinated in moist paper toweling for 18 hours. A 1:10 ratio (w/v) of scutella to buffer was used. The slurry obtained by maceration is centrifuged at 30,000 × g for 20 min and the supernatant obtained electrophoresed for 20 hours at 20 ma current in vertical starch gels in a Buchler apparatus, using methods previously described (Hart 1975).

At the completion of electrophoresis the gel is sliced vertically precisely at the level of the bottom of the slots. The portion of the gel below the level of the slots is discarded. The cut surface of the remainder of the gel is stained for GOT (Hart 1975) until the GOT-3 bands become visible, at which time the strain is discarded and the gel thoroughly rinsed with distilled H_2O. The blocks of starch which contain the electrophoretically fast and slow isozymes are then excised from the remainder of the gel and frozen for 2 hours at -20°C. The blocks of starch are then thawed, the stained portions of the starch removed and discarded, and the remainder of the starch extruded twice through a needleless 2 ml syringe. The starch pastes are then centrifuged at 30,000 x g for 20 min, after which aliquots of the two supernatants, of appropriate quantities so that they contain approximately equal amounts of GOT-3 activity, are mixed and placed in dialysis tubing. The mixture is dialysed against 250 volumes of 0.1 M sodium acetate buffer, pH 5.0, containing 40 m moles pyridoxal 5' phosphate. Dialysis is continued for 4 hours with a change in the dialysis buffer at 2 hours. All operations are carried out at 2-5°C. Following dialysis, the mixture is electrophorresed in disc polyacrylamide gels, at the completion of which the gels are stained for GOT activity. The electrophoretic and GOT staining procedures have been described previously (Hart 1975).

Three types of references samples were prepared and routinely included in the experiments which utilized isozymes eluted from starch gels. One consisted of a mixture prepared as described above, except for the use of a 0.1 M potassium phosphate buffer, pH 6.0, rather than a 0.1 M acetate buffer, pH 5.0, for dialysis. The second consisted of eluates containing individual isozymes obtained from the starch gel blocks which were dialysed against the pH 5.0 buffer without mixing of the eluates. The third consisted of an extract of the selected strain that was prepared immediately prior to polyacrylamide gel electrophoresis by the method used in the earlier genetic studies of the Triticinae (Hart 1975).

A total of about 0.4 ml of extract was placed in the 10 slots of the starch gels from which, after electrophoresis, isozymes were eluted. With the amphiploid line and the chromosome addition strain, sufficient quantities of the electrophoretically fast and slow GOT-3 isozymes for one recombination experiment were obtained from one starch gel. However, the eluates obtained from two starch gels were required for each experiment with the *T. turgidum* strain.

Results and Discussion

Representative results of experiments with the nulli-3A tetra-3D derivative of the cultivar 'Chinese Spring' of hexaploid wheat and strain G919 of the A genome diploid species *T. monococcum* are shown in Fig. 1. The *T. monococcum* GOT-3 has an electrophoretic mobility coincident with that of GOT-3c of 'Chinese Spring'. Genetic studies (Hart 1975) have provided evidence that GOT-3c of 'Chinese Spring' is composed of $\alpha^3\alpha^3$ di-

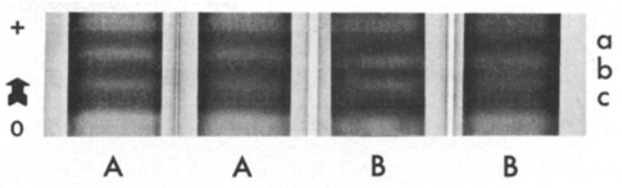

Fig. 1. GOT-3 zymogram phenotypes produced by electrophoresis of mixtures of extracts obtained from the nullisomic-3A tetrasomic-3D derivative of the cultivar 'Chinese Spring' of *T. aestivum* and strain G919 of *T. monococcum*. The letters on the right side of the figure identify the locations of the GOT-3 isozymes. Migration was toward the anode from the origin, as indicated by the arrow. Extracts were prepared in a 0.1 M potassium phosphate buffer containing 40 m moles pyridoxal 5' phosphate as described in Materials and Methods. (A) Extracts were prepared in a pH 6.0 buffer. (B) Extracts were prepared in a pH 7.0 buffer

mers and GOT-3a (the enzyme expressed by the nulli-3A tetra-3D strain) of $\beta^3\beta^3$, $\delta^3\delta^3$, and $\beta^3\delta^3$ dimers. No evidence of a structural difference between β^3 and δ^3 has been obtained. They have been distinguished only on the basis of their genetic site of origin. A mixture of extracts of the *T. monococcum* and nulli-3A tetra-3D strains subjected to the subunit recombination procedure generates three GOT-3 isozymes (Fig. 1, A). One isozyme, the electrophoretically fast form, corresponds in electrophoretic mobility to GOT-3a, a second to the *T. monococcum* isozyme (thus also to GOT-3c of 'Chinese Spring'), and the third is of intermediate electrophoretic mobility, coincident with 'Chinese Spring' GOT-3b. When the amounts of GOT-3 activity contributed to the mixture by the two strains are approximately equal, the relative staining intensities of the three zones of GOT-3 activity approximate a 1:2:1 ratio, the binomial proportions that should be attained at equilibrium if random association of subunits into equally active dimers occurs. Identical treatment of individual extracts of the two strains produces no detectable change in the enzymes - a single form with unaltered electrophoretic mobility is observed in each gel. Zymograms produced by electrophoresis of a mixture incubated at pH 7.0 express predominantly the electrophoretically fast and slow GOT-3 forms (Fig. 1, B). Only after prolonged exposure of gels to the GOT stain can the form of intermediate mobility be detected.

These results indicate that the active GOT-3 enzymes contained in crude tissue extracts dissociate

Table 1. Schematic model for the subunit composition of the active GOT-3 isozymes of *T. turgidum* cultivar 'Rolette', the 'Chinese Spring'-'Imperial' amphiploid, and the *T. aestivum-A. elongatum* chromosome 3Ag addition line (The expected quantitative distribution of the isozymes is indicated by the ratios preceding the dimers.)

Isozymes	*T. turgidum* cv. 'Rolette'	'Chinese Spring'-'Imperial' Amphiploid	*T. aestivum-A. elongatum* Addition Line
GOT-3a	$\frac{1}{4}$ $\beta^3\beta^3$	$\frac{1}{4}$ $\beta^3\beta^3$, $\delta^3\delta^3$, $\beta^3\delta^3$	$\frac{1}{4}$ $\beta^3\beta^3$, $\delta^3\delta^3$, $\beta^3\delta^3$
GOT-3b	$\frac{2}{4}$ $\alpha^3\beta^3$	$\frac{2}{4}$ $\alpha^3\beta^3$, $\alpha^3\delta^3$, $\rho^3\beta^3$, $\rho^3\delta^3$	$\frac{2}{4}$ $\alpha^3\beta^3$, $\alpha^3\delta^3$, $\varepsilon^3\beta^3$, $\varepsilon^3\delta^3$
GOT-3c	$\frac{1}{4}$ $\alpha^3\alpha^3$	$\frac{1}{4}$ $\alpha^3\alpha^3$, $\rho^3\rho^3$, $\alpha^3\rho^3$	$\frac{1}{4}$ $\alpha^3\alpha^3$, $\varepsilon^3\varepsilon^3$, $\alpha^3\varepsilon^3$

into protomers at pH 6.0 and that random reassociation of the dissociated protomers into active enzymes occurs when the pH of the medium is elevated into the alkaline range. No attempt has been made to determine precisely the pH at which reassociation occurs. The results obtained by incubation of mixtures at pH 7.0 suggest that most protomers will be associated in active enzymes at pH 7.0. The generation of one new form of intermediate electrophoretic mobility in these experiments supports the hypothesis that the active enzymes are dimers. $\alpha^3\beta^3$ and $\alpha^3\delta^3$ dimers are predicted to have an electrophoretic mobility intermediate between that of $\alpha^3\alpha^3$ and the three types of dimers produced by β^3 and δ^3 (Hart 1975).

Recombination of the subunits of GOT-3 isozymes by incubation at pH 5.0 (in a 0.1M sodium acetate buffer) and by incubation at pH 8.0 in a phosphate buffer, otherwise using the procedure described in Materials and Methods, was attempted. The former was successful but the level of recovery of the three GOT-3 forms was relatively much less than occurred following incubation at pH 6.0. No evidence for dissociation and recombination of subunits was obtained following incubation at pH 8.0.

The cultivar 'Imperial' of *S. cereale* expresses a GOT-3 enzyme with an electrophoretic mobility coincident with that of GOT-3c of 'Chinese Spring' and with the GOT-3 enzyme of *T. monococcum* strain G919. Each of the experiments described above that was performed with nulli-3A tetra-3D and the *T. monococcum* strain was also performed with nulli-3A tetra-3D and 'Imperial' and the same results obtained. These findings provide further support for the hypothesis that the active GOT-3 enzymes are dimers. They also provide strong support for the proposal, advanced by Tang and Hart (1975) on the basis of genetic evidence, that the GOT-3 isozymes expressed by the 'Chinese Spring'-'Imperial' amphiploid and the 'Chinese Spring'-'Imperial' chromosome 3R addition line are the products of random associations of four types of protomers, namely, ρ^3, produced by Got-R3 of 'Imperial', and α^3, β^3, and δ^3. Dissociation and recombination of the subunits of the $\rho^3\rho^3$ dimers of 'Imperial' and the $\beta^3\beta^3$, $\delta^3\delta^3$, and $\beta^3\delta^3$ dimers of the nulli-3A tetra-3D strain would be expected to produce a form of intermediate mobility composed of $\beta^3\rho^3$ and $\delta^3\rho^3$ dimers.

A schematic model, derived from genetic studies of the Triticinae (Hart 1975; Tang and Hart 1975; Hart et al. 1976), for the subunit composition of the active GOT-3 isozymes of the cultivar 'Rolette' of *T. turgidum*, the 'Chinese Spring'-'Imperial' amphiploid, and the *T. aestivum-A. elongatum* chromosome 3Ag addition line is shown in Table 1. Dissociation and recombination of the subunits of GOT-3a and GOT-3c of each of these strains was attempted using isozymes eluted from starch gels after electrophoresis of crude extracts. In each case, after incubation at pH 5.0, three GOT-3 isozymes were generated, corresponding in electrophoretic mobility to GOT-3a, -3b, and -3c, and in each case when GOT-3a and GOT-3c contributed approximately equal amounts of activity to the incubated mixture, the relative staining intensities of the three zones of GOT-3 activity approximated a 1:2:1 ratio. No change was detected in the electrophoretic properties of eluted GOT-3a and GOT-3c isozymes which were individually incubated, without mixing, in the pH 5.0 buffer. A single form with unaltered electrophoretic mobility was observed in each gel. These results are consistent with

predictions generated by the model shown in Table 1 and thus constitute strong support for that model.

Recombination of the subunits of eluted GOT-3a and GOT-3c isozymes by incubation of mixtures of these isozymes at pH 6.0, pH 7.0, and pH 8.0 (in a 0.1 M potassium phosphate buffer), otherwise using the procedure described in Materials and Methods, was attempted. No evidence for dissociation and recombination of subunits was obtained following incubation at these pH's.

The hypotheses of subunit structure and genetic control of the Triticinae GOT's that were originally derived from genetic studies are strongly supported by the results of the several experiments reported here. Similar in vitro studies of other higher plant GOT's have not been reported. A procedure developed for the in vitro dissociation and recombination of the subunits of the alcohol dehydrogenase (ADH) isozymes of *Triticum* (Hart 1971) has been extensively used in studies of the ADH's of several species, including maize (Fischer and Schwartz 1973; Freeling 1974; Schwartz 1975), sunflower (Torres 1974a, 1974b, 1976), and humans (Smith et al. 1973). It appears probable that the simple procedure reported here for the dissociation and recombination of the subunits of GOT isozymes may also be useful in studies of the related enzymes of other species.

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