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Active Site Labeling with Dansyl-Glutamyl-Glycyl-Arginyl Chloromethyl Ketone Demonstrates the Full Activity of the Refolded and Purified Tissue-Type Plasminogen Activator Variant BM 06.022

ULRICH KOHNERT,* MANFRED WOZNY, MANUEL LLINAS, ANITA ROOS, AND STEPHAN FISCHER

Boehringer Mannheim GmbH, Biochemical Research Center Penzberg, Nonnenwald 2, D-82377 Penzberg, Germany

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ABSTRACT

BM 06.022 is a tissue-type plasminogen activator deletion variant that is comprised of the kringle 2 and the protease domain of the native molecule. BM 06.022 is expressed as inactive inclusion bodies in *E. coli* and transferred into the active enzyme by an in vitro folding process. Active site labeling with dansyl-glutamyl-glycyl-arginyl chloromethyl ketone provides evidence that the purified BM 06.022 is fully active and that misfolded species are completely removed by affinity chromatography on ETI-Sepharose. The comparison of the kinetics of the inhibition of BM 06.022 with that of CHO-t-PA indicates that the active centers of both enzymes are rather similar. The further evaluation of the site of interaction of BM 06.022 and DnsEGRck by mass spectroscopy and amino acid sequence analysis revealed that the inhibitor is bound selectively to His_{322}, which is part of the catalytic triad of this serine protease.

Index Entries: t-PA; variant; *E. coli*; refolding; active site labeling.

*Author to whom all correspondence and reprint requests should be addressed.

Abbreviations: CHO-t-PA, recombinant tissue-type plasminogen activator from transformed Chinese hamster ovary cells; DnsEGRck, dansyl-glutamyl-glycyl-arginyl chloromethyl ketone; MUGB, 4-methylumbelliferyl-*p*-guanidino benzoate.

INTRODUCTION

BM 06.022 (Reteplase [recommended international nonproprietary name]) is a tissue-type plasminogen activator (t-PA) variant comprised of the kringle 2 and the protease domain of t-PA (*1*). The DNA of BM 06.022 comprises the part of the tPA cDNA coding for amino acids 1–3 and 176–527 (numbering according to [*2*]). BM 06.022 is expressed as inclusion bodies in *E. coli* and is transferred into its active form by an in vitro folding process. Purification of BM 06.022 is achieved by affinity chromatography on Erythrina trypsin inhibitor (ETI) immobilized to CNBr-Sepharose^R. Currently, BM 06.022 is being evaluated as a fibrinolytic agent for the treatment of acute myocardial infarction. The plasma half-life of BM 06.022 is longer by a factor of four as compared with CHO-t-PA (*3*). Because of the reduced plasma clearance, BM 06.022 is administered by iv bolus injection, which results in good early patency of the infarct-related arteries (*4*).

In order to prove that the refolded and purified BM 06.022 is completely in the active form, we employed the method of inhibition of serine proteases by chloromethyl ketones to determine the active site concentration of this enzyme. This irreversible inhibition of serine proteases has been valuable in the characterization of various enzymes. In this article, we demonstrate the active site labeling of BM 06.022 with the irreversible inhibitor dansyl-glutamyl-glycyl-arginyl chloromethyl ketone (DnsEGRck). DnsEGRck has been shown to form a 1:1 complex with CHO-t-PA (*5*). Furthermore, our data support a specific interaction of DnsEGRck with His_{322} of the catalytic triad.

MATERIALS AND METHODS

BM 06.022 was produced as inclusion bodies in *E. coli*. After in vitro folding, the active BM 06.022 was isolated by affinity chromatography as described in ref. *1*. CHO-t-PA was isolated from the supernatant of CHO cells by affinity chromatography on Red Sepharose (*6*) and ion-exchange chromatography on TSK-SP (*7*). The two-chain form of both enzymes was produced by incubating both enzymes with plasmin-Sepharose as described in ref. *1*. DnsEGRck was obtained from Calbiochem, Bad Soden, Germany. All other reagents were of analytical grade, and were obtained either from Boehringer Mannheim, Mannheim, Germany or from Sigma, Munich, Germany.

Purification of DnsEGRck

DnsEGRck was dissolved in water (5 mg/ml) and purified by reversed-phase HPLC gradient elution (solution A: 0.13% [v/v] TFA/water, solution B: 0.1% (v/v) TFA in 80% acetonitrile/20% water, flow rate: 0.7 mL/min, separating gradient: 0–55% B within 24 min, column: Eurospher C8, 4.6 × 250 mm, Knauer, Germany). The eluting peptides were monitored at 220 nm. The purity of the fractions was checked by plasma desorption mass spectrometry on a Bioion 20 mass spectrometer.

Inhibition of BM 06.022 and CHO-t-PA with DnsEGRck

BM 06.022 and CHO-t-PA were incubated with DnsEGRck as described in ref. *5*. Briefly, BM 06.022 and CHO-t-PA were adjusted to 1 and 1.5 mg/mL, respectively, with 0.5*M* Arg/H_3PO_4, pH 7.2. Five-hundred microliters of the plasminogen activators were incubated with 50 μL of various concentrations of DnsEGRck at 37°C for 30 min. The residual amidolytic activity was determined according to ref. *1*.

Determination of the Inhibitory Efficiency (k_2/K_i)

The single-chain and the two-chain (tc) forms of BM 06.022 and CHO-t-PA were incubated with DnsEGRck at 37°C (0–210 s). The molar ratio of enzyme to inhibitor was 1:11, 1:22, and 1:33 for BM 06.022 (tc), CHO-t-PA (tc), and CHO-t-PA and 1:33, 1:44 and 1:55 for BM 06.022. The residual amidolytic activity was determined according to ref. *1*. The inhibitory efficiency k_2/K_i was calculated according to the method of Kitz and Wilson (*8*) from Eq. (1).

$$1/k_{app.} = K_i/k_2 * 1/[\text{DnsEGRck}] + 1/k_2 \quad (1)$$

k_{app} was calculated from Eq. (2).

$$\ln E/E_t = -k_{app.} * t \quad (2)$$

E = units of the enzyme after incubation with DnsEGRck, and E_t = total units of the enzyme added to the assay.

Tryptic Digest BM 06.022 Inhibited by DnsEGRck

DnsEGRck inhibited BM 06.022 was produced by incubation with DnsEGRck at a molar ratio of 1:2 for 30 min at 37°C. Two-hundred and fifty micrograms of the inhibited BM 06.022 were concentrated to 10 μL with a speed vac concentrator. The concentrated sample was resuspended in 200 μL of resuspension buffer (100 m*M* Tris-HCl, pH 8.5, 0.3% [w/v] EDTA, 6*M* guanidinium hydrochloride, 0.01% Tween 80) containing 10 m*M* DTE and incubated at 37°C for 30 min. One-hundred microliters of resuspension buffer containing 100 m*M* iodoacetic acid were added, and the samples were incubated at 37°C for 30 min. Following the addition of 4

μL mercaptoethanol, the samples were thoroughly dialyzed against 100 m*M* Tris-HCl, pH 8.5. Five-hundred microliters of the dialyzed sample were mixed with 10 μL of the trypsin solution (25 μg of trypsin in 50 μL of 1% [v/v] acetic acid). Tryptic digestion was performed by incubation at 37°C for 16 h. The digests were stopped by addition of 20 μL of 10% (v/v) trifluoro acetic acid (TFA) and separated by reversed-phase HPLC gradient elution (solution A: 0.13% TFA/water, solution B: 0.10% TFA in 80% acetonitrile/20% water, flow rate: 0.5 mL/min, separating gradient: 0–65% B within 103 min, column: Eurospher C8, 4.6 × 250 mm, Knauer, Germany). The eluting peptides were monitored at 220 nm. Additionally, the DnsEGRck-containing peptides were selectively detected by measuring the fluorescence of the HPLC eluent with excitation and emission wavelengths of 340 and 530 nm, respectively.

Analysis of the BM 06.022–DnsEGRck Complex and the DnsEGRck-Containing Peptide by Mass Spectroscopy

Samples were desalted by reversed-phase chromatography, dried in a speed vac concentrator, and prior to application to the mass spectrometer, redissolved in a 1:1 mixture of methanol and water containing 1% (v/v) formic acid. The instrument used was a Vestec VT 201 mass spectrometer.

N-terminal Sequence Analysis

N-terminal sequence analysis was performed on an automated system (ABI 473 A equipped with analysis system 610 A) using standard procedures.

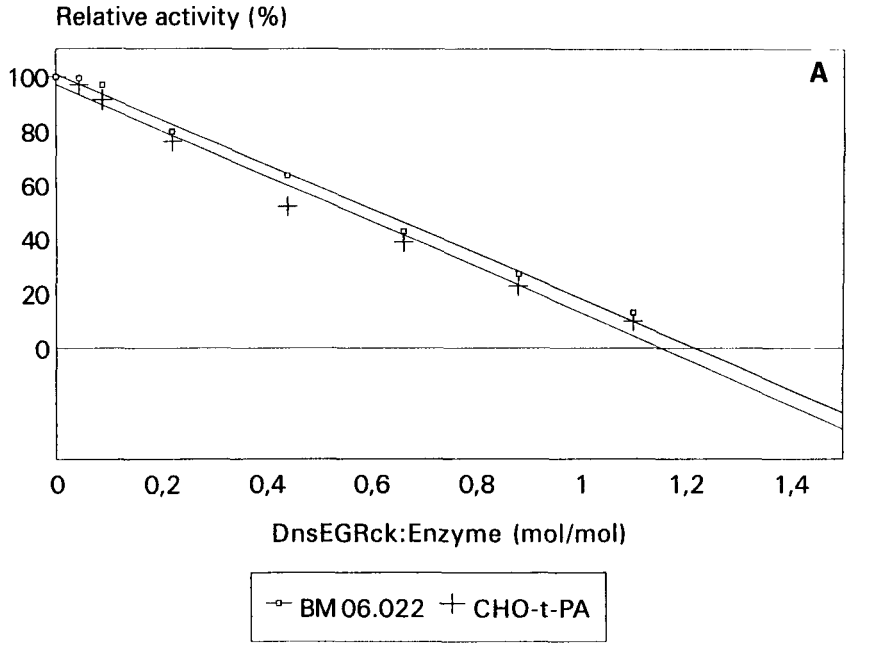
RESULTS

Inhibition of BM 06.022 and CHO-t-PA with DnsEGRck

The single-chain and the two-chain forms of BM 06.022 (BM 06.022 [tc]) and CHO-t-PA (CHO-t-PA [tc]), were incubated with increasing amounts of DnsEGRck. Data presented in Fig. 1 reveal a linear decrease of the amidolytic activity of both enzymes. Although the single-chain forms of both enzymes were completely inhibited at a DnsEGRck:enzyme ratio of 1:1.2–1:1.3, inhibition of the two-chain forms of both enzymes was achieved at a molar ratio of 1:1.1.

Kinetics of the Incorporation of DnsEGRck

The two-chain forms of BM 06.022 and CHO-t-PA were incubated in the presence of three different concentrations of DnsEGRck. The rate of inactivation of both enzymes was determined by measuring the residual



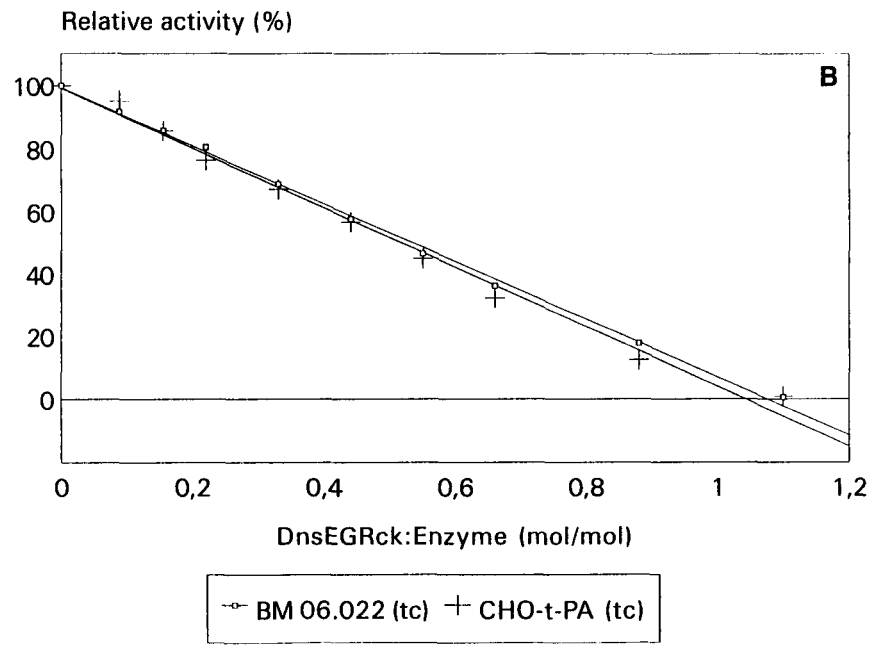


Fig. 1. Inhibition of BM 06.022 and CHO-t-PA by DnsEGRck. Variable amounts of DnsEGRck were incubated with the single-chain (**A**) and the two-chain forms (**B**) of BM 06.022 and CHO-t-PA as described under Materials and Methods. The residual activity was determined with S 2288 as substrate.

Table 1
Inhibition of BM 06.022 (tc) and CHO-t-PA (tc) by DnsEGRck

Protein	k_2, min^{-1}	K_i, μmol/L	k_2/K_i, L/μmol/min
BM 06.022 (tc)	2.9 ± 0.7	12.5 ± 4.3	0.23
CHO-t-PA (tc)	1.2 ± 0.1	3.8 ± 0.5	0.32

Table 2
Inhibition of BM 06.022 and CHO-t-PA by DnsEGRck

Protein	k_2, min^{-1}	K_i, μmol/L	k_2/K_i, L/μmol/min
BM 06.022	0.60 ± 0.1	60.7 ± 2.9	0.010
CHO-t-PA	0.35 ± 0.15	29.9 ± 10.0	0.011

amidolytic activity, k_2/K_i were 0.23 and 0.32 L/μmol/min for BM 06.022 (tc) and CHO-t-PA (tc), respectively. The binding constants (K_i) were 12.5 and 3.8 μmol/L and the rate constants (k_2) were 2.9 and 1.2 min^- for BM 06.022 (tc) and CHO-t-PA (tc), respectively (Table 1).

The single-chain forms of BM 06.022 and t-PA were inhibited by DnsEGRck in a very similar manner. The inhibitory efficiencies (k_2/K_i) were 0.010 and 0.011 L/μmol/min, respectively. K_i was 60.7 and 29.9 μmol/L and k_2 was 0.60 and 0.35 min^- for BM 06.022 and CHO-t-PA, respectively (Table 2). These data reveal that the inhibition of the single-chain form of both enzymes is less effective by a factor of 24–31 as compared to the respective two-chain form.

Analysis of the DnsEGRck-Inhibited BM 06.022 by Mass Spectroscopy

The molecular weight of the BM 06.022–DnsEGRck complex was determined by electrospray mass spectroscopy. The main signals detected corresponded to a mol wt of 40,159.5 ± 2.0 Dalton. This value is in good agreement with the theoretical value of 40161.2 for BM 06.022 covalently modified with a single DnsEGRck molecule. Within the limit of detection, neither unmodified BM 06.022 nor BM 06.022 labeled with two DnsEGRck molecules was detectable (Fig. 2).

Determination of the DnsEGRck Binding Site in BM 06.022

Figure 3 shows the tryptic peptide map of a DnsEGRck-modified BM 06.022 sample. From the fluorescence signal of the sample, the labeled

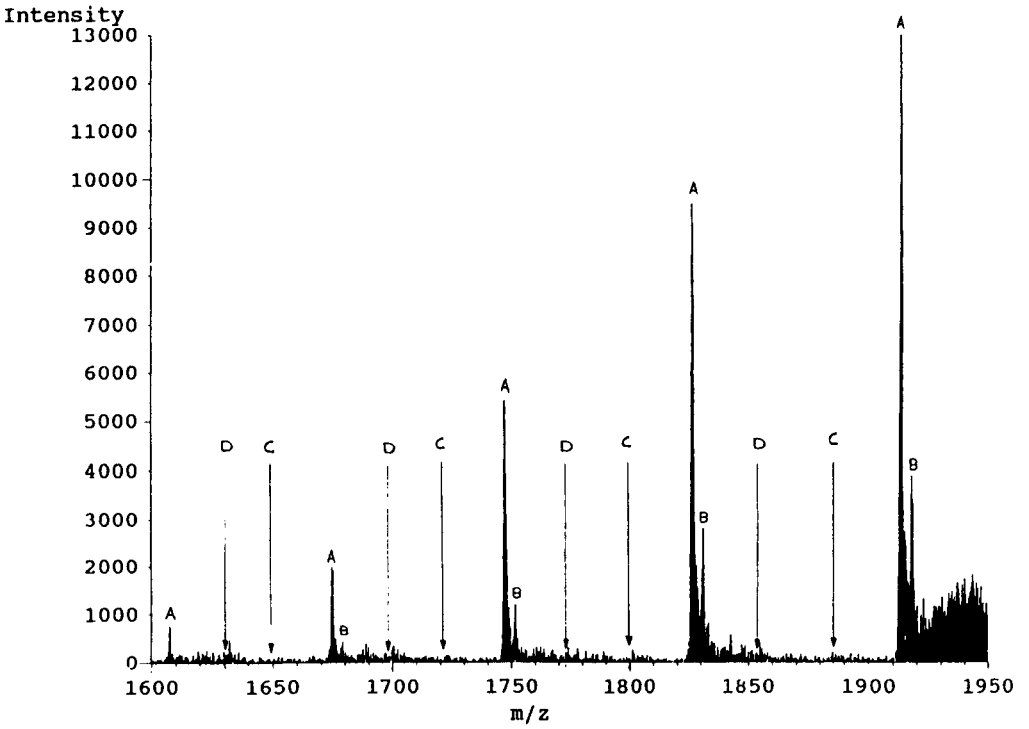


Fig. 2. Electrospray mass spectrum of BM 06.022 incubated with an excess amount of DnsEGRck. BM 06.022 was incubated with twofold excess of DnsEGRck. The inhibited protein was purified by RP-HPLC and analyzed by electrospray mass spectroscopy. (**A**) Signals corresponding to BM 06.022 with covalently linked DnsEGRck; (**B**) signals corresponding to BM 06.022 with covalently linked DnsEGRck and noncovalently linked phosphate; (**C**) expected locations of signals corresponding to underivatized BM 06.022; (**D**) expected location of signals corresponding to BM 06.022 derivatized with two DnsEGRck molecules.

peptide can be clearly identified. The mol wt of the peptide was determined as 3318.4 ± 0.6 Dalton by electrospray mass spectroscopy. These data indicate that the DnsEGRck-containing peptide corresponds to amino acids 305–327 of t-PA (expected mol wt 3318.8 Dalton). The N-terminal part of the amino acid sequence of this peptide was determined by automated Edman sequencing. The cycle corresponding to amino acid 322 showed no amino acid signal. However, all other cycles revealed the amino acids expected from the BM 06.022 sequence. This indicates that His_{322} is modified by DnsEGRck. The other fluorescence peaks present in Fig. 3 are either owing to incomplete tryptic cleavage of BM 06.022 or to some remaining impurities in the DnsEGRck preparation (purity about 90% as determined by plasma desorption mass spectrometry).

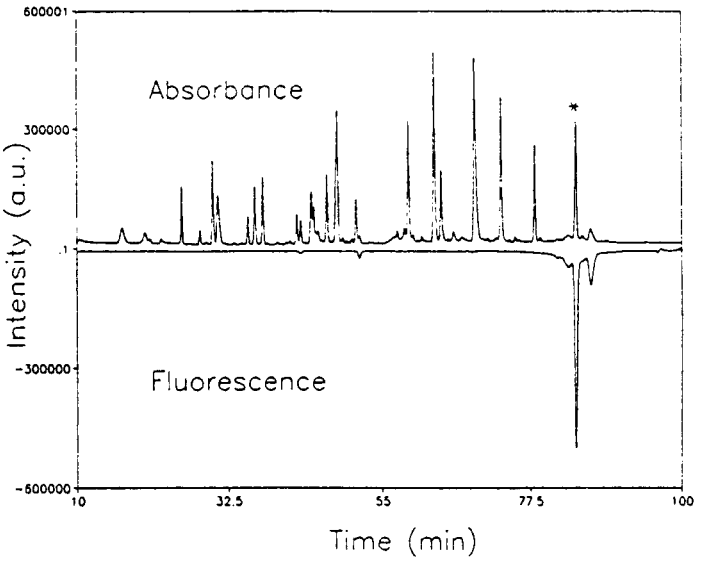


Fig. 3. Tryptic pattern of BM 06.022 inhibited by DnsEGRck. BM 06.022 was inhibited by a two-fold excess of DnsEGRck. The inhibited protein was purified by RP-HPLC and incubated with trypsin as described under Materials and Methods. The tryptic peptides were separated by RP-HPLC. Top: absorbance at 220 nm; bottom: fluorescence intensity (excitation at 340 nm, emission at 530 nm).

DISCUSSION

In vitro folding of proteins from inclusion bodies leads not only to the correctly structured molecule, but also to the formation of aggregates and of inactive species that have to be removed during chromatographic purification (*9*). The t-PA deletion variant BM 06.022 is expressed as inclusion bodies in *E. coli*, and purification is achieved by affinity chromatography on ETI-Sepharose.

The concentration of active BM 06.022 and the removal of inactive species can only be quantified by active site titration. 4-Methylumbelliferyl-*p*-guanidino benzoate (MUGB), 4-amidino-2-nitrophenyl 4'-anisate, and DnsEGRck are described in the literature as titrants for t-PA (*5,10,11*). The 4-amidino-2-nitrophenyl 4'-anisate is not commercially available. Titration with MUGB and extrapolation to infinite MUGB concentration resulted in only 70% activity of the two-chain forms of BM 06.022 and CHO-t-PA (data not shown). However, the analysis of the cleavage of several low-mol-wt substrates by BM 06.022 and CHO-t-PA yielded similar values for K_m and k_2 as published in the literature for t-PA (*12*). Furthermore, the cleavage of MUGB is very dependent on the exact assay conditions (*10*). t-PA and BM 06.022 are poorly soluble. The solubility and functional stability can be increased by the addition of arginine (*13*). In order to avoid any aggregation of the enzymes all measurements were carried out in the presence of arginine. It may be speculated that the guanidino group

of arginine may influence the reaction of the enzymes with MUGB. Therefore, the rather low activity of both enzymes against MUGB may be the result of the assay conditions, rather than the presence of inactive species.

BM 06.022 and CHO-t-PA are inhibited by DnsEGRck in a similar fashion. Although the complete inhibition of the single-chain form of both enzymes is achieved at a DnsEGRck/enzyme ratio of 1:1.2–1:1.3, the two-chain forms are completely inhibited at a ratio of 1:1.1. Regarding the 90% purity of DnsEGRck, the molecular DnsEGRck/enzyme ratio for complete inhibition of the single- and the two-chain forms is about 1:1.1 and 1:1, respectively.

The inhibition of the single-chain form of BM 06.022 indicates that the single-chain form is an active enzyme rather than an inactive precursor like chymotrypsinogen. The increased activity of the two-chain form against DnsEGRck may reflect some structural rearrangements on cleavage of the Arg_{275}—Ile_{276} bond, which are also the reason for the higher catalytic efficiency of the two-chain form of BM 06.022 and t-PA against several low-mol-wt substrates (*12*).

The kinetic analysis of the inhibition reveals a good agreement between BM 06.022 and CHO-t-PA. Although K_i and k_2 for the two-chain form of t-PA are rather similar to the data published by Higgins and Lamb (*5*), k_2/K_i for the single-chain form of t-PA differ by a factor of 2.3. The reason for this difference is not clear. However, it may be speculated that arginine may in particular influence the inhibition of the single-chain form of t-PA by DnsEGRck.

The stoichiometry of the BM 06.022–DnsEGRck complex was 1:1 as determined by mass spectroscopy. The tryptic digestion of the modified enzyme and the amino acid sequence analysis of the DnsEGRck-containing peptide reveal that the inhibition of BM 06.022 by DnsEGRck is the result of a specific binding to His_{322} of the catalytic triad. The high reactivity of the active site histidine against chloromethyl ketones is typical for all serine proteases and is the result of the complex interaction with several amino acids (*14*). This structural feature is maintained in the refolded BM 06.022.

In conclusion, our data provide evidence that the in vitro folding and purification of the t-PA deletion variant BM 06.022 from inclusion bodies yield only active molecules with an active site whose structure closely resembles the catalytic center of t-PA. The labeling with DnsEGRck allows a correct quantification of the active molecules present in the preparation of BM 06.022.

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