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Pitfalls in screening streptococci for retrieving superior streptokinase (SK) genes: no activity correlation for streptococcal culture supernatant and recombinant SK

Malihe Keramati · Farzin Roohvand · Mohammad Mehdi Aslani · Fatemeh Motevalli · Shohreh khatami · Arash Memarnejadian

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Abstract Streptokinase (SK), the heterogeneous protein family secreted by some groups of β-hemolytic streptococci (βHS), is a plasminogen activator and well-known drug for thrombolytic therapy. Differences in plasminogen activation property of streptococcal culture supernatants (SCS) have been traditionally used to identify superior producer strains and SK genes (*skc*) for recombinant SK (rSK) production. However, the role of SK heterogeneity and whether SK activities in SCS correlate with that of their corresponding rSK is a matter of debate. To address these concerns, SCS of nine group C streptococci (GCS) screened among 252 βHS clinical isolates were compared for plasminogen activation using S-2251 chromogenic assay. The GCS (*Streptococcus equisimilis*) showing the highest (GCS-S87) and lowest (GCS-S131) activities were selected for PCR-based isolation of *skc*, cloning and rSK production in *Escherichia coli*. The 6×His-tagged rSK proteins were purified by NI–NTA chromatography, analyzed by SDS-PAGE and Western blotting and their activities were determined. While SCS of GCS-S87 and GCS-S131 showed different plasminogen activations (95 and 35 %, respectively) compared to that of the reference strain (GCS-9542), but interestingly rSK of all three strains showed close specific activities (1.33, 1.70, and 1.55×10^4 IU mg^{-1}). Accordingly, SKS87 and SKS131 had more than 90 % sequence identity at the amino acids level compared to SK9542. Therefore, SK heterogeneity by itself may not contribute to the differences in plasminogen activation properties of SCS and evaluation of this activity in SCS might not be a proper assay for screening superior *skc*.

Keywords Plasminogen activation · Plasmin amidolytic substrate · Recombinant streptokinase · Streptococcus equisimilis

M. Keramati · M. M. Aslani
Microbiology Department, Pasteur Institute of Iran, Tehran, Iran

M. Keramati · F. Roohvand (✉) · F. Motevalli · A. Memarnejadian
Hepatitis and AIDS Department, Pasteur Institute of Iran, Tehran, Iran
e-mail: farzin.roohvand@gmail.com; rfarzin@pasteur.ac.ir

S. khatami
Biochemistry Department, Pasteur Institute of Iran, Tehran, Iran

Introduction

Streptokinase (SK), the potent human plasminogen activator, is a 414-residues (47-kDa) protein secreted by β-hemolytic streptococci (βHS) groups A, C, and G (GAS, GCS, GGS, respectively). SK converts the inactive blood plasminogen to the active (fibrinolytic) enzyme plasmin that degrades fibrin and fibrinogen (the primary proteins of a blood clot) [17]. Prior studies indicated that plasminogen activation property (SK activity) of streptococci culture supernatants (SCS) vary considerably among SK-producing strains, ranging from extremely low to high fibrinolytic activities [25, 28]. SK is a heterogeneous protein with the overall sequence identity between any pair of molecules (from different streptococci) ranging from 80 to 98 % [11, 20]. There is also significant heterogeneity of the SK even in the same streptococcal group [11].

The detailed structure studies of SK demonstrated that it consists of three domains of almost similar size and independent folding (termed α, β and γ in order from N to C terminus of the polypeptide) that have different structural-function properties [26]. Among SK domains, β (residues:

151–290) shows the highest degree of polymorphism and several SK variants have been classified based on the polymorphism in the major variable region (residues 147–218) of SK-β domain [13]. Although SK polymorphism [28] or crucial amino acid changes in its protein sequence [5] have been linked to the functional differences in plasminogen activation [21, 29], their definite roles in this process is still controversial. Additional regulatory factors [7, 25], streptococcal genetic background [19] and co-presence of other streptococcal proteins [21] have been also proposed for the different SK activities observed in SCS.

SK is the first and most widely used fibrinolytic (thrombolytic) agent for the treatment of diverse circulatory disorders including myocardial infarction [17]. Among SK-producing streptococci GCS strains, which are less fastidious in growth requirements and produce lower amounts of erythrogenic toxin, were the favorite group for production of SK as a therapeutic agent [24]. In this context, the first and most widely used strain for commercial SK production was a group C *Streptococcus equisimilis* H46A (ATCC 12449), which was selected (more than half a century ago) among other SK-producing streptococci for its high plasminogen activation activity in culture supernatant [17]. Although the potential risk of bleeding and allergic reactions had reduced the popularity of SK for thrombolytic therapy in the last decade, the results of several clinical trials on various thrombolytic agents suggested that SK is the most cost-effective fibrinolytic drug and almost as safe as its other counterparts such as tissue plasminogen activator (tPA) [4]. Therefore, SK is particularly the drug of choice for thrombolytic therapy in many poor economies and developing countries. In this context, and to explore for a superior SK for thrombolytic therapy, a number of national screening or mutation/selection studies have been carried out [1, 2, 8]. In all of these studies, quantifying the plasminogen activation properties of SCS was the basis of the screening method to identify and select the most active SK producer among wild clinical isolates or mutant strains. Due to the pathogenicity of SK natural host, low yields and streptococcal toxin contaminations however, recombinant production of SK in heterologous protein expression systems is currently the explored method for its commercial production [24]. Accordingly, genes coding for SK (*skc*) from *S. equisimilis* H46A and GCS-9542 (*skc*-2) as standard sources of commercial production of recombinant SK (rSK) [22] have been also expressed in several heterologous hosts [17]. Since SK activity in SCS might be a complex feature contributed by a number of regulatory factors besides the *skc* polymorphism itself [19], this argues against the efficacy of screening procedures (for selection of the superior SK-producing strains based on SK activity in SCS) for the aim of gene isolation and rSK production. Therefore, weather a SK-producing streptococcal strain that has been selected for its highest SK activity in its culture supernatant would also provide a superior *skc* for heterologous rSK production is a matter of debate and has not been addressed before. To address this concern, in the present study, the SK activities of SCS of selected strains of GCS (which showed significantly different activities) were compared with that of their recombinant forms. To the best of our knowledge, this study represents the first attempt to draw a conclusion on the validity of screening methods for selection of the SK-producing streptococci for isolation of the superior SK genes and production of rSK with higher activities in relation to SK heterogeneity.

Materials and methods

Bacterial strains and growth conditions

Nine strains of GCS investigated in this study (Table 1) were screened from our departmental collection of 252 streptococcal clinical isolates from different patients during 2006–2010. Morphological characteristics of the strains were confirmed according to Bergey's Manual of Systematic Bacteriology [9]. Serotyping was performed by using specific antiserum group C (Mast, UK) as a gold standard and GCS subspecies were biochemically characterized by fermentation test of 10 % ribose, sorbitol, lactose, and trehalose solutions (Table 1) [9]. *Streptococcus equisimilis* ATCC 9542 [3] was used as reference strain throughout this study. The pQE30 plasmid and M15 *Escherichia coli* cells (Qiagen, USA) were used for cloning and expression of the SK genes and DH5α *E. coli* cells were used for propagation of plasmids. GCS strains were grown in blood agar and Todd-Hewitt Broth (THB) (Difco, USA) and *E. coli* cells were cultured in Luria–Bertani media.

Plasminogen activation assay for quantification of SK activity

The typical colorimetric method, which is based on plasmin amidolytic substrate S-2251, a synthetic tripeptide (H–D-Val-Lue-Lys-p-nitroaniline) (Sigma, USA) was used to measure the plasminogen activation activity of both GCS culture supernatants and their corresponding rSK proteins [30]. In brief, the culture supernatant of GCS strains in THB medium were harvested by centrifugation (cell-free culture fluids) at mid-log phase (OD_{600} of 0.6–0.7) and filtered using a 0.22-μm filter (Whatman, Germany). Subsequently, 20 μl of the filtered fluids was incubated with 100 μl human plasminogen (20 μg ml^{-1}, Tris–HCl 50 mM

Table 1 Source of GCS strains in this study and their characterization

No.	Sample code	Culture source[a]	Disease	Fermentation test[b]				Subgroup of GCS
				Ribose	Sorbitol	Lactose	Trehalose	
1	GCS-S04	T.C	Pharyngitis	+	−	v	+	*S. equisimilis*
2	GCS-S05	T.C	Pharyngitis	+	−	v	+	*S. equisimilis*
3	GCS-S08	H.V	Puerperal Fever	+	−	v	+	*S. equisimilis*
4	GCS-S87	T.C	Pharyngitis	+	−	v	+	*S. equisimilis*
5	GCS-S91	T.C	Acute Tonsillitis	+	v	+	−	*S. dysgalactiae*
6	GCS-S131	H.V	Puerperal fever	+	−	v	+	*S. equisimilis*
7	GCS-K17	T.C	Acute Tonsillitis	+	−	v	+	*S. equisimilis*
8	GCS-K19	T.C	Acute Tonsillitis	+	−	v	+	*S. equisimilis*
9	GCS-K34	T.C	Acute Tonsillitis	+	−	v	+	*S. equisimilis*

[a] *T.C* Throat culture; *H.V* Human vagina; (+) Positive results; (−) Negative results; (*v*) Variable

[b] After identification of GCS via serology by specific antiserum group C (Mast, UK), subspecies were biochemically characterized by fermentation test of 10 % ribose, sorbitol, lactose, and trehalose solution

pH7.4) at 37 °C for 15 min. At the end of incubation time, S-2251 substrate (20 µl of 2.5 mM) was added to the mixture, and absorbance at 405 nm (in a microplate reader, BioHIT, UK) was monitored every 5 min for a total period of 60 min [21]. All experiments were performed in duplicate in the presence and absence of plasminogen. Culture supernatants of the reference strain (*S. equisimilis* ATCC 9542) and intact TBH media (not utilized for culturing) were used as positive and negative controls of the assays, respectively. Culture supernatants were also evaluated for presence of any plasminogen-independent hydrolysis of the substrate to confirm the accuracy of the assay. Serial dilutions of Streptase® (CSL, Behring, Marburg, Germany), a commercial SK, were used to prepare the standard curve for SK activity. Optical density (OD)at 405 nm was plotted against time and activity rate (slope) was determined from linear portion of the curve. The standard curve was used to calculate the Unit/ml activities of the GCS culture supernatants.

For quantitative assay of rSK, purified and refolded proteins (5 nM each) were added separately to the assay buffer (50 mM Tris–Cl, pH 7.4) containing: S-2251 (1 mM) and plasminogen (1 µM). The reaction was continuously monitored at 405 nm and the change of absorbance was measured as a function of time (*t*) at 22 °C. SK activities were obtained from the slopes of the activation progress curves based on changes of the absorbance against time (OD/t^2) [30].

Cloning of streptokinase genes

Based on the results of the plasminogen activation assay for SCS (Fig. 1), bacteria showing the highest (GCS-S87) and the lowest (GCS-S131) activities were selected for retrieving the SK gene and recombinant production of SK. Genomic DNA of these two strains and that of the reference strain (GCS-9542) was isolated by using bacterial genomic extraction kit (Axygene, USA). SK genes coding DNA sequences (excluding the N-terminal signal sequences) corresponding to the mature polypeptide were amplified by PCR from genomic DNA using the designated forward (*Bam*HI-skf: 5′TGGATCCATTGCTGGACCT GAGTGGCTG3′) and the reverse (*Pst*I-skr: 5′CGCCGC AGTTATTTGTCGTTAGGGTTATC3′) primers. In-frame cloning of the PCR amplicons into the *Bam*HI/*Pst*I digested pQE30 vector (in separate reactions), downstream of the vector derived N-terminal polyhistidine $(His)_6$ sequence resulted in the construction of chimeric pSK9542, pSK87, and pSK131plasmids harboring *skc* of GCS-9542, S87, and S131, respectively (Fig. 2a). All cloning techniques were done according to the standard procedures [23]. The resultant constructs were confirmed by the appropriate restriction enzyme digestions and DNA sequencing analyses.

Protein expression, purification, and renaturation

The competent *E. coli* M15 cells were transformed with the confirmed constructs (pSK9542, pSK87, and pSK131 in separate reactions) for protein expression. Expression of *skc* was induced by the addition of 1 mM isopropyl-*β*-D-thio-galactoside (IPTG) at mid-log phase. After 6–7 h of growth at 37 °C, cells were harvested by centrifugation. The bacterial pellet was suspended in 15 ml of lysis buffer (20 mM Tris–Cl [pH 8.0]–100 mM NaCl–8 M urea) per 50 ml of original culture volume, sonicated briefly, and clarified by centrifugation. The clarified supernatant was applied to NI–NTA agarose column (Qiagen, USA), which was equilibrated in lysis buffer. The column was washed twice with washing buffer (20 mM Tris–Cl [pH

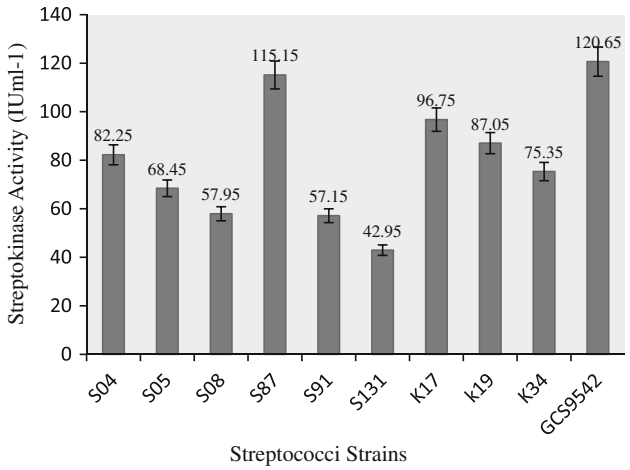

Fig. 1 Plasminogen activation analysis of nine screened GCS culture supernatants (S04 to K34) with that of reference strain (GCS-9542). The activities were measured by S-2251 chromogenic method as detailed under the experimental procedure. *Error bars* indicate the standard division of three independent experiments

6.3]–100 mM NaCl–8 M urea). The bound protein was eluted from the resin with pH gradient of elution buffer (20 mM Tris–Cl [pH 5.9–4.5]–100 mM NaCl–8 M urea). Purified rSK proteins were refolded by dialysis against refolding buffer (20 mM Tris–Cl, pH 7.4, 10 % glycerol) for 14–18 h at 4 °C [6]. The final concentration of rSK was determined by measurement of optical densities at 280 nm (OD_{280}).

SDS-PAGE and Western-blot analyses

The homogeneity of the expressed SK proteins was determined by SDS-PAGE and confirmed by Western-blotting assays according to the standard procedure [23]. Gels were either stained with Coomassie blue (for SDS-PAGE analyses) or equilibrated in phosphate buffer saline (4.3 mM Na_2HPO_4–1.4 mM K_2HPO_4 [pH 7.4]–137 mM NaCl) for 10 min and transferred to a 0.45-mm

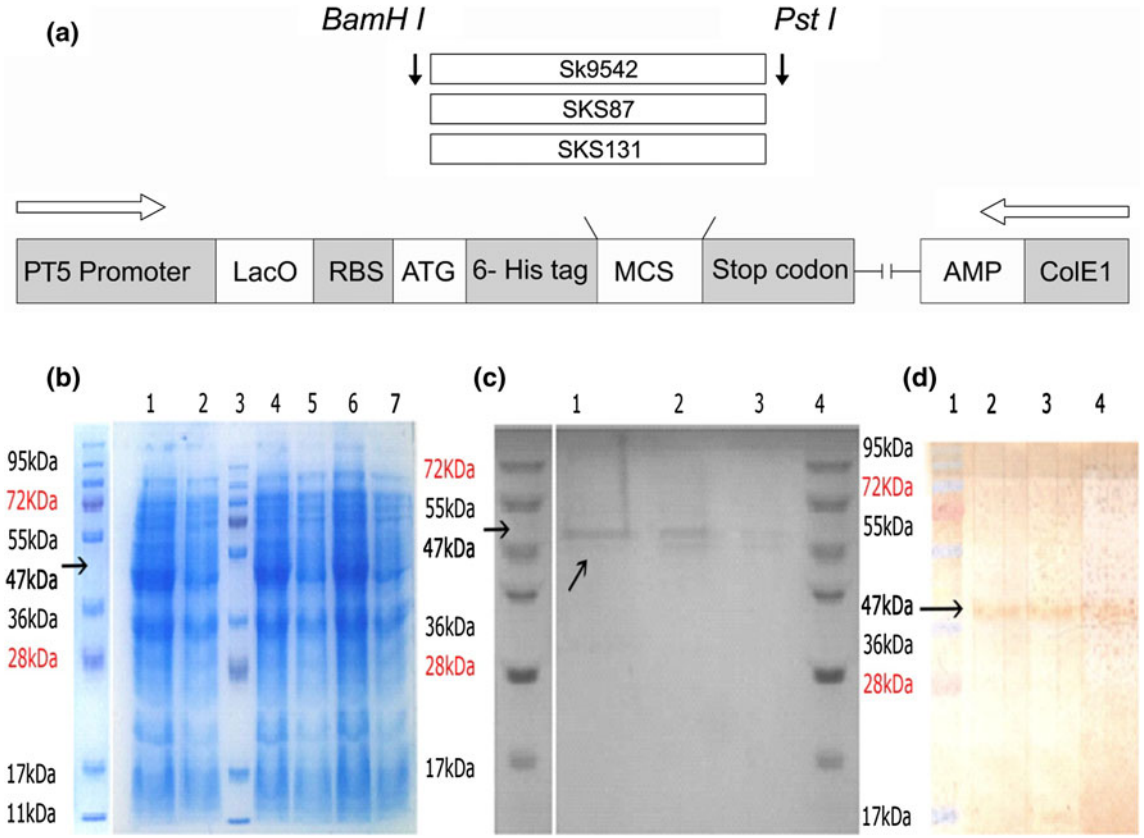

Fig. 2 Cloning and expression of SK in *E. coli* M15. **a** Schematic representation of the recombinant vector, pQE30 harboring SK gene. PCR-amplified SK genes were inserted into *Bam*H1/*Pst*I sites of MCS. PT5, LacO, RBS, and ATG denote T5 promoter, lactose operator, ribosome binding site, and translation initiation code (ATG), respectively. **b** SDS-PAGE analysis of rSK expression. *Lanes 2, 5,* and *7*: whole-cell lysate of M15 *E. coli* cells harboring pSK87, pSK131, and pSK9542 constructs, respectively, before induction for expression; *Lanes 1, 4,* and *6*, the same cells 6 h after induction by 1 mM IPTG. **c** SDS-PAGE analysis of purified rSK. *Lane 1*: rSKS87, *lane 2*: rSKS131 and *lane 3*: rSK9542. **d** Western-blot analysis of rSK proteins, *Lane 2*: rSKS87, *lane 3*: rSKS131 and *lane 4*, rSK9542. *Lanes 3* in b, *4* in c, and *1* in d correspond to protein molecular weight markers. The position of 47- and 44-kDa rSK protein fragments are shown by *arrows*

nitrocellulose membrane (Bio-Rad, USA) for Western-blot analysis. Recombinant proteins were detected using mouse anti-His monoclonal antibody (Qiagen, USA). Horseradish peroxidase (HRP)-conjugated goat anti-mouse antibody (Qiagen, USA) was used as secondary antibody. Protein bands were finally visualized by brief exposure to 3, 3-diaminobenzidine (DAB, Sigma, USA).

Statistical analyses

Differences of SK activities among streptococcal strains were determined using unpaired, two-tailed Student's *t* test with 95 % confidence intervals. Statistical analysis was carried out using SPSS software version 16.0 (SPSS, Inc., Chicago, IL).

Sequence alignment of the SK proteins and analyses

The nucleotide sequences of the SK genes reported here have been deposited in the GenBank nucleotide sequence database. The *skc* from GCS-S87 to GCS-S131 strains have been assigned accession numbers HQ913573.1 and HQ913575.1, respectively. Multiple alignment of deduced amino acid sequences of SK from *S. equisimilis* 9542, S87, and S131 was performed using molecular evolutionary genetic analysis and sequence alignment software (MEGA4) [27].

Results

GCS strains and plasminogen activation analyses of their culture supernatants

From the 252 initially screened bacterial samples, βHS including group A, C, and G were found in 75 out of 252 specimens and the remaining samples mostly belonged to group B (109 out of 252) and D (36 out of 252) streptococci. Group A was found in 65 βHS streptococci specimens followed by GCS (9) and GGS (1), respectively. Distribution of the investigated GCS in different clinical samples and their serogroups are shown in Table 1. Among nine GCS employed in this study, eight belonged to *S. equisimilis* and one to *S. dysgalactiae* subgroups of streptococci. Bacterial growth curves of all examined GCS strains and that of the reference strain (GCS-9542) in THB media, under identical growth conditions, were almost the same (data not shown). Culture supernatants of all streptococci were collected at midlog phase (OD_{600} of 0.6–0.7), approximately 3–4 h after incubation (inoculation) and promptly utilized for plasminogen activation assay. Culture supernatants of examined GCS demonstrated significant differences in plasminogen activation (SK activities) ranging from 42 to 120 IU ml^{-1} (Fig. 1). None of the culture supernatants showed plasminogen-independent hydrolysis of the substrate, indicating the specificity of the assay. GCS-S87 and GCS-S131, which showed the highest and lowest plasminogen activation activities in their culture supernatants, respectively, (115.2 vs. 42.9 IU ml^{-1}), were selected for retrieving the SK gene and recombinant production of SK (Table 2) and were compared with that of the reference strain (GCS-9542), which showed the highest level of SK activity in culture supernatant (120 IU ml^{-1}) (Fig. 1).

Expression and purification of recombinant SK

Constructions of chimeric pSK9542, pSK87, and pSK131 plasmids for expression of SK were confirmed by restriction enzyme digestions and DNA sequencing analyses (results not shown). The recombinant SK proteins of selected strains (rSKS87, rSKS131) and that of the reference strain (rSK9542) were expressed in *E. coli* M15. An expected band of approximately 47 kDa corresponding to the mature SK protein was observed in total cell lysate of each recombinant *E. coli* (Fig. 2b). The purification of rSK, which contained a vector-derived, N-terminal polyhistidine tag, was carried out by Ni_2+ -chelating affinity chromatography. Results showed that the expression level for the three cloned *skc* genes ranged from 0.12 to 0.18 mg ml^{-1} for the purified fractions (Table 2). SDS-PAGE (Fig. 2c) and Western-blot (Fig. 2d) analyses of the purified fractions of the rSK proteins also indicated the presence of a minor 44-kDa band corresponding to the intermediate SK products, which has been previously proposed to be caused by C-terminal deletion of 31 amino acid residues of the native SK [15].

Table 2 Plasminogen activation analysis of SCS and recombinant streptokinase (rSK)

Strain	SK activity of culture supernatant (IU ml^{-1})	Relative activity	rSK activity (x10^4 IU ml^{-1})	Total purified rSK (mg ml^{-1})	Specific activity of rSK (x10^4 IU mg^{-1})	Relative activity
GCS-9542	120.65 ± 1.50[a]	1	0.28 ± 0.07	0.18	1.55 ± 0.09	1
GCS-S87	115.15 ± 1.40	0.95	0.16 ± 0.04	0.12	1.33 ± 0.04	0.85
GCS-S131	42.95 ± 0.25	0.36	0.14 ± 0.10	0.14	1.70 ± 0.12	1.1

[a] Values shown are the mean of at least three independent experiments

Plasminogen activation of rSK compared to the streptococcal culture supernatant

As shown in Fig. 1, the plasminogen activation activity of the streptococcal culture supernatants (SCS) of the GCS-S87 (115.2 IU ml^{-1}) and GCS-S131 (42.9 IU ml^{-1}) presented about 95.4 and 35.5 % activity of the GCS-9542 reference strain (120.7 IU ml^{-1}), respectively. Interestingly however, as presented in Table 2, all three recombinant proteins (rSKS87, rSKS131, and rSK9542) corresponding to the same GCS strains (GCS-S87, GCS-S131, and GCS-9542) showed close specific activities for plasminogen activation (1.33, 1.70, and 1.55×10^4 IU mg^{-1}, respectively).

Amino acid homology of SK proteins

Nucleotide and deduced amino acid sequence of the cloned SK genes were analyzed. Amino acid alignment of the three SK genes revealed that SKS131 had 99.3 % amino acid homology with SK9542 (Fig. 3). In fact, only three residue substitutions (R45Q, N228 K, and F287I) could be detected for these two SK proteins. SKS87, however, represented a higher degrees of amino acid variations compared to SK9542 (about 94 % amino acid homology) with a total of 27 residue substitutions. Most of these amino acid variations (12 residues out of 27) were in SK α-domain (1–150 aa) and other substitutions were distributed in different regions of the SKγ (291–414) and SK β- domains.

Discussion

In the present study, only nine GCS could be identified in the total of 75 GAS, GCS, and GGS isolates among the initially screened 252 βHS. The low rate of GCS screening in our clinical isolates is in accordance with the low incidence of this serotype in human streptococcal infections as previously reported [12]. In addition, *S. equisimilis* was the major subspecies of GCS in our samples, which is also consistent with previous observations [12]. The SK activity in bacterial culture supernatants of the screened GCS strains was determined by plasminogen activation assay [21]. Plasminogen activation levels considerably varied among GCS strains (Fig. 1). These results are in complete agreement with prior reports in which considerable variety of SK activity in SCS among SK-producing streptococci (ranging from lack of activity for some GAS [11] to 996 IU ml^{-1} for some GCS mutant strains) were reported [1]. Whether the differences in plasminogen activation properties of different SCS are due to a higher level of SK expression and secretion by the streptococcal strains (i.e., the role of SK promoter and transcription/expression regulatory factors) [19] or SK heterogeneity and polymorphism (i.e., differences in SK amino acid sequences in different strains) [21] or both is a matter of debate [25]. Concerning the first possibility, prior studies indicated that a rather complex system including both *cis* and *trans*-acting factors might be involved in control/regulatory transcription of SK [20]. The *cis*-acting elements include the

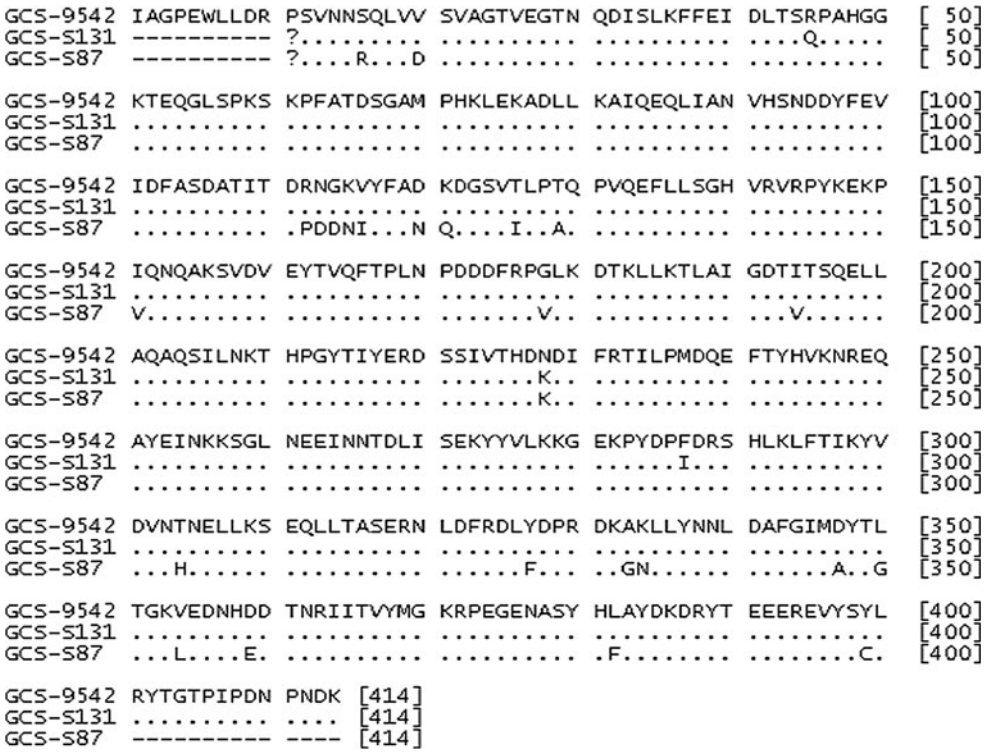

Fig. 3 Amino acid sequence alignment of SK proteins corresponding to SKS131, SKS87, and SK9542. The alignment was created using MEGA4 software [27]. The sequences accession numbers are HQ913575.1 and HQ913573.1 for skc from *S. equisimilis* GCS-S131 and GCS-S87, respectively. Conserved (identical) amino acids in the alignment are indicated by *dots*. The question mark "*?*" denotes nucleotides sequences that are not precisely identified by sequencing reaction data

core promoter and the promoter-upstream region, which is located in an intrinsic DNA bending locus [10]. While SK core promoter regions of different strains are almost conserved, upstream regions might be required for regulated expression of SK through interaction of *trans*-acting factors. The *trans*-acting factors that are proposed for control of SK expression involve the two-component signal transduction system of CovRS (as a negative transcription regulator) and FasA (as a positive transcription regulator) [16]. Therefore, any functional differences (due to mutations) in these *cis*/*trans*-acting regulatory factors (i.e., genetic background of the streptococci besides heterogeneity of SK gene itself) may affect SK expression/secretion by streptococci and ultimately determine the total SK activity of the streptococcal culture supernatant. In fact, the higher production of SK by GCS H46A is linked to an amber mutation in the CovR gene that inhibits the repressor function of this regulator, while a specific mutation in the upstream region of SK gene in GAS NZ131 enhances the affinity of CovR, thereby repressing the SK expression in this streptococci [25]. Obviously, however, in heterologous expression of SK (in *E. coli*), the only streptococcal factor for SK activity of the purified recombinant protein would be SK gene sequence and its heterogeneity [7, 25]. Concerning the second possibility, while involvement of discrete regions and crucial amino acids in the functional domains of SK have been described to affect its plasminogen activation properties were described [5, 29] other studies, however, have indicated that at least sequence polymorphism in the SK-β domain (the most polymorphic domain of SK) might not affect the activity of SK [18].

In the present study, we aimed to compare the SK activity of SCS of GCS with that of their recombinant forms and to draw a conclusion on the potential role of *skc* heterogeneity for SK activity. To address this concern, strains representing the lowest (GCS-S131) and the highest (GCS-S87) plasminogen activation levels in their culture supernatants were selected for retrieving the SK gene and recombinant production of SK. Results of plasminogen activation analyses showed that despite the significant variations for SK activity in SCS, all recombinant SK proteins (rSKS87, rSKS131, and rSK9542) showed close specific activities (Table 2). These results clearly indicated that the primary sequence of SK genes of explored GCS strains in our study might not be the major reason for different plasminogen activation levels in the culture supernatants of these strains. Evaluation of the deduced amino acid sequences of the cloned SK genes indicated three residues substitutions in case of SKS131 (R45Q, N228 K, and F287I) compared to SK9542 (Fig. 3). However, there is no prior report on involvement of residues 228 and 287 of SK in plasminogen activation and they seemed to bear no functional relevance. On the contrary, residues 41–48 of the SK α-domain have been reported to play a critical role in plasminogen substrate recognition [14]. However, as could be expected, the plasminogen activation property of SKS131 was not affected by substitution of Arg 45 with another charged amino acid (Gln) in this critical α-domain region. SKS87, the other cloned SK with a total of 27 residue substitutions, represented higher degrees of amino acid variations compared to SK9542 (Fig. 3). The majority of these substitutions, however, did not present any functional relevance. Previous studies based on random mutagenesis demonstrated the importance of residues 315–340 of SKγ-domain in substrate plasminogen activation [31]. In contrast to this prior report, variation of three residues in this segment (residues 315–340 of SKγ-domain) did not critically affect the plasminogen activation properties of SKS87 in our study (Fig. 3 and Table 2).

Collectively, in the present study, for the first time, (to our best of knowledge) by cloning and expression of SK genes from selected GCS strains, which showed significantly different plasminogen activation properties in their culture supernatants, we could clearly show that besides *skc* heterogeneity, other curial and host-dependent factors influence the SK activity of the SCS in GCS strains. In addition, we could identify a number of amino acid residues substitutions in different regions of *skc* that may not critically affect SK activity. Our results argue against the efficacy of currently employed screening procedures (which is based on SK activity of culture supernatants) to screen superior SK-producing strains [1, 2, 8] for the final aim of gene isolation and rSK production.

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Conflict of interest The authors declare that they have no conflicts of interest.

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